Corrigenda for MSc Thesis

"Physiology and Ultrastructural Aspects of
Adventitious Abscission in Internodal Explants of
Impatiens sultani, *Impomoea batatas* and *Begonia corallina*"

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The following corrections should be noted in this thesis.

Page	Line	Correction
2	18	Change "... the level of IAA" to "reduced levels of IAA"
2	19	Change "... is thought" to "... are thought"
3	4	Delete "of" after "promote"
8	12	Change "coordinate" to "coordinates"
8	13	Change "his review" to "this review"
9	10	Add "and McManus" after "Osborne"
10	3	Change "zeatin (0.1 g^{-1})" to "zeatin (0.1 $g.l^{-1}$)"
11	8	Add "(Flow Laboratories A'asia Pty Ltd North Ryde, NSW 2113, Australia) after "detergent"
12	23	Change "Propark" to "Porapak" Add "(length 1.22 m, ID 3 mm)" after "column"
19	5	Change "grater" to "greater"
21	17	Change "he" to "the"
27	12	Change "buteric" to "butyric"
Following 27	Table 2 Legend	Add after final word "abscission" the words "Results from 2-4 experiments except treatment marked with asterisk (*) which is from one experiment with 10 replicates"
Following 27	Tables 4,5 Legends	Change "seperation" to "separation"
Following 27	Table 9 Legend	Change "different time intervals" to "different times"

Page	Line	Correction
29	8	Add "IAA induced and" after "attributed to"
35	14	Change "That" to "that"
37	1	Change "ethylene" to "Ag^{+}"
37	2	Change "and" to "an"
37	10	Change "results" to "result"
Following 41	Tables 3-6 Legends	Change "seperation to "separation"
47	11, 12	Change "phase" to "face"
62	1	Add "Oxidase" after "Cytochrome"
62	21	Change "µM" to "µmol"
63	11	Add "(Single Vial Reagent)" after "S.V.R."
68	6	Add "within minutes" after "were able"
Following 77	Fig. 6 legend	Add "in upper segments" after "V_{alt}"
85	20	Change "Valdovinus" to "Valdovinos"
87	18	Change "Abscissic" to "Abscisic"
88	5	Change "sheding" to "shedding"
88	7	Change "Defferal" to "Deferral"

Certified correct according to examiners' recommendations.

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Physiological and Ultrastructural Aspects of Adventitious Abscission in Internodal Explants of *Impatiens sultani, Ipomoea batatas* and *Begonia corallina*

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Statement

This thesis describes my own original work.

D. P. Dharmawardhana

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ABSTRACT

Internodal explants of *Impatiens sultani*, *Ipomoea batatas* and *Begonia corallina* when cultured develop a transverse separation layer, 3-8 mm above the base. Inclusion of IAA in the culture medium is known to increase the frequency and regularity of abscission.

Including zeatin in the medium with IAA accelerated abscission, with a concomitant increase in ethylene evolution just before abscission. Ethylene alone applied at 5-40 $\mu l\ l^{-1}$ also caused abscission. *Ipomoea batatas* explants form an unusual upper separation layer (especially in very young internodal explants) 0.5-5.0 mm below the upper exposed surface in addition to the lower separation layer. Ethylene synthesis inhibitor amino-ethoxyvinylglycine (AVG) and ethylene action antagonist silver thiosulphate inhibited both upper and lower separation layer development.

The abscising distal part of *Impatiens sultani* explants showed a rapid decline in the activity of cytochrome pathway of respiration, which was partly due to respiratory control by adenylates during early stages. At later stages the mitochondria demonstrated signs of deterioration at ultrastructural level. On the other hand, the basal part exhibited an increase in respiration just before abscission, coinciding with the increase in ethylene evolution. This respiratory increase was due to increased activity of the cytochrome pathway; the alternative oxidase capacity also increased but did not contribute to respiration.

Ultrastructurally, these adventitious or secondary separation layer cells were found to be very similar to normal or primary abscission zone cells, in showing increased activity of the endomembrane system during abscission. Unlike in *Impatiens sultani* and *Begonia corallina, Ipomoea batatas* showed cell division at the separation layer, at the onset of its development. The newly divided cells, being confined to the proximal fracture surface after abscission, seemed to act like a protective layer.

These findings also support the concept that most cells in these internodes have the potential to act as target cells for separation layer formation when appropriate inductive conditions occur, which are supposed to be provided by a decrease in auxin concentration in the morphologically upward direction. This in turn is thought to sensitize some cells to respond to ethylene (either exogenous or endogenous) by producing hydrolyzing enzymes that leads to cell separation and abscission.

CONTENTS

GENERAL INTRODUCTION

Abscission can be defined as separation of cells, tissues and organs from the main plant body. This may include leaves, fruits, flowers, stamens, seeds and other plant organs (Kozlowski 1973), and its occurrence is widespread in the plant kingdom (Addicott 1982). Typically, abscission is achieved by the separation of a narrow layer of cells called a separation layer found within an abscission zone. Abscission zones are usually found at the base of most discrete organs and are commonly recognizable by external differences (colour, shape), and also by anatomical and cytological distinctions. The position of such abscission zones is defined at an early stage of the organ development. After the determination of the abscission zone, its subsequent activation may take place at a later stage when the organ senesces. Thus, in such instances the abscission zone can be referred to as a region of arrested development (Addicott 1982). In adventitious abscission on the other hand, the positional definition and differentiation (activation) of the separation layer cells seems to proceed without a pause (Warren Wilson 1986). The prime characteristic of adventitious (secondary) abscission is that it can be induced by surgical, chemical or other treatments, and occur away from the typical, morphologically defined positions of normal (regular, primary) abscission. The position of the normal abscission zones, however, cannot be altered even with the harshest of experimental treatments (Sexton and Roberts 1982). The adventitious mode of abscission has been reported to occur in various plant species involving different plant parts, including leaf blades, petioles, pedicels and stems (Addicott 1982).

To study the involvement of hormones and the physiology of the abscission process, explants containing the abscission zone have been widely used. Explants provide a convenient system for experimentation since they provide large quantities of reproducible material which could be easily handled under controlled conditions, and also due to the ease of administering compounds influencing abscission. *Phaseolus vulgaris, Gossypium spp.* and *Coleus spp.* have been the most popular explants used for the study of normal abscission (Bornmann 1967; Jackson and Osborne 1972; Halliday and Wangermann 1972). Adventitious abscission studies have also used explants of *Phaseolus, Gossypium* and *Impatiens sultani* internodes and apple and pear pedicels (Chapter 1). Although it is necessary to be cautious in extrapolating results obtained from explants to *in vivo* systems (Jacobs 1962), the hormonal changes that bring about abscission in explants and whole plant organs as well as the cell biology of the two systems have been subsequently found to be somewhat similar (Sexton and Roberts 1982).

The induction of abscission is controlled by the interplay of hormonal influences and tissue sensitivities. The key hormones involved are IAA, ethylene and ABA (Addicott 1982). Auxins play a major role in the control of abscission and the level of IAA at the abscission zone (e. g. altered due to delamination or senescence of a leaf) is thought to be responsible for the activation of the separation layer in the process of normal abscission. This is supported by numerous evidence where application of IAA distal to the separation layer had inhibited abscission while proximal application has stimulated abscission process (Reviews, Jacob 1962; Addicott 1970). Similar observations have been made on adventitious abscission as

well (Pierik 1977; Warren Wilson *et al.* 1986). Warren Wilson *et al.* (1986) suggested that the position of adventitious abscission in *Impatiens sultani* internodal explants is determined above sites of high auxin by auxin acting as a morphogen.

Ethylene is found to promote of abscission in various species and plant parts, and abscission of many plant parts have been reported to accompany an increase in ethylene production (see Sexton *et al.* 1985). The auxin status of the abscission zone is considered a main factor regulating the sensitivity of the abscission zone to ethylene (Riov and Goren 1979; Abeles 1973). Ethylene can act directly on the sensitized cells of the separation layer, inducing them to produce cell wall hydrolytic enzymes or act indirectly by aiding in reducing the supply of auxin reaching the abscission zone (Sexton *et al.* 1985; Chapter 1). ABA is also known to stimulate abscission by its ability to stimulate ethylene production (Jackson and Osborne 1972; Addicott 1982), although, there are some reports of direct action of ABA without affecting ethylene production (Marynick 1977; Dorffling *et al.* 1978). Ethylene inhibitors and antagonists have been used widely to investigate the involvement of ethylene on abscission (Chapter 2).

In both explants and intact organs the part distal to the abscission zone often demonstrates signs of yellowing or senescence before abscission (Smillie 1962; Beevers 1976). The senescence in leaves is considered as a regulated process. Although it is mainly a catabolic process involving increased activity of hydrolytic enzymes, it is also reported to involve some anabolic processes such as *de novo* synthesis of proteolytic enzymes (Tetley and Thimann 1974; Brady and Tung 1975). The breakdown and loss of nucleic acids and proteins (Woolhouse 1967; Thomas

and Stoddart 1980) are the the most basic of all senescence related events in that these changes could underlie most of the physiological changes during senescence.

Ultrastructural changes that have been reported associated with senescence include the reduction of ribosomes and disorganization of the chloroplasts (Butler and Simon 1971). The mitochondria by contrast usually remain structurally intact until later stages of senescence. The endoplasmic reticulum and dictyosomes also disappear, while the tonoplast often disintegrates, the plasmalemma remains intact until final stages of senescence.

Reports on changes in respiratory properties during senescence are controversial. Some report that respiratory activity drops gradually during later stages of senescence (Smillie 1962; Beevers 1976). However, in many senescing organs (especially in detached organs) an increase in respiration is commonly observed during senescence (Solomos and Laties 1974b; Romani 1987). Ethylene is also found to be capable of producing similar climacteric like increase in respiration in many types of tissues (Rhodes 1980). The increase in respiration has some times been attributed to the involvement of cyanide resistant respiration (Solomos and Laties 1974b). Thimann *et al.* (1977) proposed that more than 75% of this rise was due to uncoupling of respiration from phosphorylation while about 25% was due to increased availability of free amino acids and sugars from hydrolytic breakdown of proteins and carbohydrates. Romani (1984, 1987) has suggested that the respiratory climacteric is largely a homeostatic response to compensate for the degradative effects of incipient programmed cellular senescence. This is also supposed to be accelerated by the autocatalytic ethylene production which becomes an added stress.

For many years the anatomy and ultrastructure of normal abscission zones have been studied (Valdovinos *et al.* 1968; Sexton and Roberts 1982). In a majority of plant species abscission is achieved by the separation of separation layer cells due to cell wall and middle lamella dissolution. Ultrastructurally, these separation layer cells typically demonstrate high endomembrane activity which is presumably involved in the production and secretion of hydrolytic enzymes that degrade the cell walls (Chapter 3).

The present investigation was initiated in the view of understanding the following:

(1) The influence of IAA, zeatin, and ethylene on the process of adventitious abscission in internodal explants of *Impatiens sultani*, *Ipomoea batatas*, and *Begonia corallina* explants (Chapter 1).

(2) The importance of ethylene in the process by the use of ethylene inhibitors (Chapter 2).

(3) The anatomical and ultrastructural details of the separation layer of adventitious abscission and to what extent it compares with that of normal abscission (Chapter 3).

(4) The respiratory characteristics of the upper senescing and lower seemingly non-senescing part of the internodal explant undergoing adventitious abscission (Chapter 4).

CHAPTER ONE

Influence of IAA, zeatin, and ethylene on adventitious abscission in internodal explants

1. INTRODUCTION

'Adventitious' type of abscission differs from 'normal' abscission (e.g. leaf fruit or flower abscission), by the separation layer being not pre-differentiated and also because it often occurs away from a recognizable abscission zone. This mode of abscission has been reported to occur in various parts of several species, including stem abscission of *Phaseolus vulgaris* (Webster and Leopold 1972), *Impatiens sultani* (Lloyd 1914; Gortner Harris 1914) and *Gossypium hirsutum* (Bornmann *et al.* 1968), and pedicel abscission of *Malus silvestris* and *Pyrus communis* (Pierik 1977, 1980). *Ipomoea batatas* and *Begonia corallina* internodes have also been found to abscise when cultured in appropriate media (J. Warren Wilson and P. M. Warren Wilson personal communications).

Detailed physiological and biochemical studies have been carried out on normal abscission in whole plant organs (Jackson and Osborne 1972, Gaspar *et al.* 1978, Riov and Goren 1979, Riov *et al.* 1986). However, similar studies on adventitious abscission have been limited. Warren Wilson *et al.* (1986) reported that *Impatiens sultani* internodes when cultured in sterile media, underwent adventitious abscission by the differentiation of a transverse separation layer a short distance away from the base of the explant. Both the rate and the frequency of abscission were found to increase with the addition of sucrose and indole- 3-acetic acid (IAA) into the incubating medium at appropriate concentrations (0.0001% IAA being the optimum). The position of the separation layer could also be modified experimentally, e.g. by increasing the concentration of IAA provided in the medium, distance of the separation layer from the base of the explant could be increased. Pierik (1980) also

found that IAA application could induce adventitious abscission in apple and pear pedicel explants and that the position of the separation layer could also be changed by changing the IAA concentration. He also reported that cytokinins could similarly stimulate adventitious abscission. Internodal abscission in *Phaseolus vulgaris* is reported to occur when explants are treated with ethylene (Webster and Leopold 1972). *Phaseolus vulgaris* pedicels could also be induced to form adventitious abscission zones by the application of IAA to the separated distal surface of the normal abscission zone and by maintaining it in an ethylene atmosphere (Osborne and McManus 1986).

Although it is still not certain whether ethylene is an essential inducer of abscission, there is a considerable body of evidence to support that it accelerates and coordinate abscission in various species, both in intact organ abscission and also in explant systems (Sexton *et al.* 1985). In his review of a number of studies which showed a positive correlation between ethylene level and abscission, most plant material exhibited an increase in ethylene production preceding abscission or there was a significant difference in the ethylene evolution between abscising and non-abscising populations. The involvement of ethylene has also been demonstrated by removal of ethylene from the environment (Jackson and Osborne 1970; Young and Meredith 1971) and by the use of ethylene synthesis inhibitors and antagonists (Sagee *et al.* 1980; Sipes and Einset 1982; Van Meeteren and de Proft 1982; Kushad and Poovaiah 1984).

The abscission accelerating effect of ethylene has been reported to occur in a number of different ways (Sexton *et al.* 1985). It can act directly by influencing

processes leading to cell wall breakdown at the abscission zone (i.e. by increasing the production of cellulases and polygalactouronases), or by causing cell enlargement at the fracture plane (Wright and Osborne 1974). Ethylene can also act directly by influencing the concentration and transport of other growth regulators which in turn can alter the abscission process. Auxin exhibits a number of interactions with ethylene during abscission, which include inhibition of transport (Beyer and Morgan 1971, Riov and Goren 1979), increased destruction and conjugation (Riov *et al.* 1982, 1986) and reduced synthesis (Ernest and Valdovinus 1971).

Most of these studies have been carried out on normal abscission zones which have pre-differentiated separation layer cells at a defined position. Osborne (1986) refers to them as committed or preprogrammed target cells which are competent to recognize and respond to ethylene, by producing hydrolases. Adventitious abscission on the other hand occurs across already differentiated tissues. Thus, the positional definition of the separation layer (i. e. the commitment to become target cells) should precede the second phase where the activation of these cells (possibly by ethylene) would occur, which brings about changes in organelles, and cell wall breakdown.

The present study attempts to understand the involvement of IAA, cytokinin and ethylene in adventitious abscission, using internodal explants of *Impatiens sultani*, *Ipomoea batatas* and *Begonia corallina*.

2. MATERIALS AND METHODS

2.1. Media Preparation

The stock solutions of IAA (1.0 g l^{-1}) and zeatin (0.1 g $^{-1}$), were prepared in 100% isopropanol and distilled water respectively. Required volumes of hormone stock solutions and sucrose were added to distilled water and the pH was adjusted to 5.5. Agar was added at a concentration of 1.0%. 5.0 ml of autoclaved media were poured into 50x25 mm scintillation vials, capped with aluminum foil and left until culture.

2.2. Plant Material

Impatiens sultani Hook., *Ipomoea batatas* (L.)Lamb. and *Begonia corallina* Carriere plants were each propagated from one parent plant by cuttings, and were grown in glasshouses with liberal fertilization. Plants were cut back approximately once a month and the new shoots were used for obtaining internodal explants.

2.3. Selection of Explants from Young Shoots

2.3.a. *Impatiens sultani*

The youngest internodes measuring more than 20.0 mm in length(usually 1-3 nodes below the first flowering node) were used (Fig. 1).

2.3.b. *Ipomoea batatas*

Only the actively elongating shoots with flexible long internodes were used. From these shoots the one below the first internode measuring more than 20.0 mm was used (Fig. 3 internode B).

2.3.c. *Begonia corallina*

From shoots emerging from the base of the plants, internodes 2-3 nodes below the first recognizable node were used (Fig. 2).

2.4. Culturing of Explants

Explants from selected internodes were cut under 1.0% 'White King' bleach (containing 4.0% available chlorine from sodium hypochlorite) with 0.4% '7X' detergent. These were surface sterilized in fresh solution of bleach for 30 minutes and rinsed in two changes of sterile distilled water for 20 minutes. They were then aseptically trimmed to 20 mm in a laminar flow, and placed vertically with morphological base down in appropriate culture media in scintillation vials. Explants were pushed into the medium to a depth of 3-4 mm and vials capped with aluminium foil. The explants were incubated at 26^{o}C in the dark. Observations were carried out every 24 hours upto 25 days.

The standard errors of percentage abscission were derived from values for each experiment while standard errors of time taken to abscission, distance to separation layer and number of roots per explant were derived from values for individual explants.

2.5. Separation Layer Position Measurement

The distance to the separation layer from the base and from the top of the explant were measured as illustrated in Fig. 4.

2.6. Removal of Ethylene from the Environment

8-10 explants cultured in appropriate media were incubated in a sealed 600ml bottle with 5ml of 10% KOH and 2ml of 0.25M $Hg(ClO_4)_2$ to absorb ethylene. Incubation bottles were uncapped and ventilated each day and KOH and $Hg(ClO_4)_2$ replaced every 2 days. Periodically an air sample from the containers was analysed by gas chromatography to ensure that an ethylene free atmosphere was maintained within the containers.

2.7. Determination of Ethylene Production During Abscission

The standard method of sealing the explant for few hours to accumulate ethylene and subsequent analysis by gas chromatography did not produce consistent results. This method could also overlook short bursts of ethylene production.

Hence the method of trapping the ethylene produced with $Hg(ClO_4)_2$ and subsequent release for measurements was used (Young *et al* 1952). Individual explants cultured as described previously were placed in a capped 250 ml bottle with two vials of 5 ml 10% KOH and 0.5 ml 0.25 M $Hg(ClO_4)_2$ solutions. Periodically the $Hg(ClO_4)_2$ solutions were replaced and the containers ventilated for 1 hour before resealing. Vials containing the absorbed ethylene were stored below 4^oC until analysis. Ethylene absorbed during incubation periods were released from the $Hg(ClO_4)_2$ complex by injecting 1.0 ml saturated of LiCl (Meir *et al.* 1985) into the vial and stored at 4^0C for 12 hours. The liberated ethylene was assayed by injecting a 1.0 ml sample into a Hewlett-Plackard 5380A gas chromatograph, equipped with a Propark R (80-100 mesh) column held at 50^oC and a hydrogen flame ionization

detector with high purity N_2 as the carrier. Results are presented on a fresh weight basis.

2.8. Use of Ethylene to Induce Abscission

Explants cultured as above in 1.0% agar medium containing sucrose (*Impatiens sultani* in 1.0% , *Ipomoea batatas* in 3.0% and *Begonia corallina* in 3.0%) were placed in 600 ml bottles with a 5.0ml of 10% KOH vial inside, and capped. Required amounts of appropriately diluted ethylene from a 100% pure ethylene cylinder were injected into the bottles through a serum stopper. Each day the bottles were opened, ventilated with fresh air, recapped and appropriate amounts of ethylene reinjected. As for other explants incubation was at 26^{o}C in the dark.

2.9. Preliminary Experiments

For the induction of abscission in *Ipomoea batatas* explants the best IAA concentration was found to be within 0.0001%-0.001% (5.7-57.0 μM) being similar to *Impatiens sultani* (Warren Wilson *et al.* 1986). *Begonia corallina*, however, required a higher IAA concentration for abscission (0.01% being the best out of 3 concentrations tested, viz 0.0001%, 0.001% and 0.01%). The frequency of abscission varied considerably in *Begonia corallina* from experiment to experiment depending on the condition of the plant material. Both *Impatiens sultani* and *Ipomoea batatas* demonstrated improved abscission in response to zeatin addition into the medium, with 0.0001% IAA. In *Begonia corallina*, however, it was not possible to establish a significant difference by the addition of zeatin (at 0.001%,

0.0001% and 0.00001% concentrations) into 0.01% IAA medium due to variation between experiments and the low percentage of abscission achieved. Provision of sucrose to the basal medium was found to be essential for abscission of *Ipomoea batatas* and *Begonia corallina* but not for *Impatiens sultani*.

All 3 species could be induced to abscise by the addition of ethylene into the environment, in the absence of any other externally applied hormone. But again, abscission in *Begonia corallina* is rather inconsistent and when it occurred the abscission layer formed 1-2mm above the base of the explant, forming a separating basal disc like segment.

3. RESULTS

3.1. Effect of Zeatin on Separation Layer Formation

3.1.1. Effect on frequency and rate of abscission

3.1.1.a. *Impatiens sultani*

Table 1 illustrates the effect of a range of zeatin concentrations (used with or without 0.0001% IAA) on abscission, from 2-7 experiments with 10-12 replicates per treatment.

Zeatin alone without IAA did not promote abscission, and both frequency and time taken from explanting to abscission were similar to results for explants cultured in control media (with only sucrose). Out of the 3 zeatin concentrations examined, in the presence of IAA, 0.0001% zeatin induced more explants to abscise (97.3%) and at a faster rate (time taken from explanting to abscission = 5.3 days). These results

were significantly different to those for explants cultured in standard abscission inducing medium with 0.0001% IAA (percentage of abscission was higher $P<0.05$, and time taken for abscission was lower $P<0.001$). The plot illustrating the progress of abscission in explants cultured in media with and without zeatin further demonstrate this stimulatory effect of zeatin on abscission (Fig. 5).

3.1.1.b. *Ipomoea batatas*

Table 2 describes results from 2-4 experiments (treatments marked with an asterisk, however, are from one experiment) with 8-10 replicates per treatment. Inclusion of zeatin in the medium (at 0.00001% or 0.0001%), in addition to 0.0001% IAA increased the percentage of abscission ($P<0.05$ in 1.0% sucrose, $P = 0.05$ in 3.0% sucrose). The time taken from explanting to abscission was also shorter in the medium where 0.0001% zeatin was added ($P<0.001$ in 3.0% sucrose). The medium with 0.0001% IAA + 0.0001% zeatin + 3.0% sucrose appeared to be the best for stimulating abscission, by having both a higher percentage of abscission, and a lower time taken to abscission. Although explants in 0.001% IAA + 3.0% sucrose medium showed a slightly lower (not significant, $P> 0.2$) time to abscission, than explants in 0.0001% IAA ± zeatin + 3.0% sucrose media, the percentages of abscission achieved in these high IAA (0.001%) media were much lower (even with the addition of zeatin).

3.1.2. Effect of sucrose concentration on frequency and rate of abscission

3.1.2.a. *Impatiens sultani*

From the results given in Table 3 (from 2-5 experiments with 10-12 replicates per treatment), the best sucrose concentration to be used with 0.0001% IAA + 0.0001% zeatin appeared to be within 1.0%-3.0%. The results varied between experiments, and in some experiments explants abscised without any sucrose in the medium. But in others the percentage of abscission was significantly lower in the absence of sucrose. This variation is likely be due to differences in the plant material used in different experiments. Based on the above experiments 1.0% sucrose was used in all abscission-inducing media in subsequent experiments.

3.2.1.b. *Ipomoea batatas*

Table 2 illustrates the effect of using 1.0% or 3.0% sucrose with different combinations and concentrations of IAA and zeatin in the culture medium on abscission. In almost all media, explants in 3.0% sucrose showed a shorter time to abscission than those in 1.0% sucrose ($P<0.001$). The only exception was 0.001% IAA + 0.0001% zeatin medium, which showed a longer time to abscission in 3.0% sucrose than in 1.0% sucrose. The latter is not very informative due to the low number of replicates and the low number of explants that abscised.

The percentage of abscission achieved in these two sucrose concentrations, however, did not differ significantly except in 0.001% IAA medium, where 3.0% sucrose medium showed a higher percentage of abscission (the number of replicates is not sufficient to demonstrate a statistically different result).

Explants could not be stimulated to abscise without sucrose in the medium, even with optimum concentrations of IAA and zeatin.

3.1.3. Effect on position of separation layer and on root formation

3.1.3.a. *Impatiens sultani*

Addition of IAA to the basal medium induced the separation layer to form further away from the base of the explant (Table 4), similar to the results obtained by Warren Wilson *et al.* (1986). Zeatin has no effect on the position of the separation layer, either when added with IAA ($P>0.3$) or when added without IAA ($P>0.5$).

As expected IAA induced root formation at the base of the explant (Table 4). Addition of 0.0001% zeatin completely inhibited root formation even in the presence of IAA. A zeatin concentration of 0.00001%, although it did not completely inhibit root formation, it did counter the stimulatory effect of IAA ($P<0.001$).

3.1.3.b. *Ipomoea batatas*

As for *Impatiens sultani*, addition of zeatin did not have any effect on the position of the separation layer (Table 5), at both IAA concentrations used ($P>0.7$). Explants cultured in the higher concentration of IAA (0.001%) formed the separation layers at a greater distance from the base than explants in lower IAA concentration (0.0001%), irrespective of zeatin addition ($P<0.001$ in medium without zeatin and $P<0.01$ in the medium with 0.0001% or 0.00001% zeatin), which was consistent with the results obtained for *Impatiens sultani* (Warren Wilson *et al.* 1986).

As in *Impatiens sultani* IAA stimulated root formation, with the higher concentration of IAA (0.001%) being more effective. Zeatin at both concentrations inhibited the root formation. Even the use of high IAA concentration (0.001%) could not counteract the inhibitory effect of zeatin on root formation.

3.2. Involvement of Ethylene During Separation Layer Formation

3.2.1. Effect of removing ethylene from the environment

3.2.1.a. *Impatiens sultani*

In two experiments with 8-12 replicates per treatment, the effect of removing ethylene from the environment (by absorbing liberated ethylene with 0.25M $Hg(ClO_4)_2$), was compared with that of controls (Table 6). The removal of ethylene from the environment of the cultured explants did not significantly affect the percentage of explants that abscised ($P>0.5$ for explants in 0.0001% IAA medium, $P>0.4$ for explants in 0.0001% IAA + 0.0001% zeatin medium). However, the time taken to abscission was slightly longer when ethylene was removed ($P = 0.1$ for explants in 0.0001% IAA medium and $P<0.01$ for explants in 0.0001% IAA + 0.0001% zeatin medium).

3.2.1.b. *Ipomoea batatas*

Results in Table 7 were from two experiments with 5-12 replicates per treatment. Removal of ethylene from the environment reduced both percentage of abscission and time taken to abscission by explants cultured in 0.0001% IAA medium ($P<0.05$ and $P = 0.02$ respectively) and in 0.0001% IAA + 0.0001% zeatin medium ($P<0.05$ and $P<0.02$ respectively). However, at the higher IAA concentration (0.001%) ethylene removal did not significantly affected the percentage or time taken to abscission.

3.2.2. Rate of ethylene evolution during abscission

3.2.2.a. *Impatiens sultani*

In both abscission inducing media, there was a prominent rise in the rate of ethylene evolution prior to abscission (Fig. 6). The peak ethylene production rate before abscission was significantly grater in the explants incubated in the medium with zeatin in addition to IAA. The time of appearance of the ethylene peak was variable between individual explants, depending on the time of abscission. Accordingly, only data from representative explants showing synchronized abscission are shown in Fig. 6 to avoid averaging of peak rates, occurring at different times.

The initial high rate of ethylene production observed (likely to be due to wound ethylene), was significantly higher in the media with IAA when compared to controls with only sucrose in the medium. Even the low basal rates of ethylene production was always lower in explants in control media.

3.2.2.b. *Ipomoea batatas*

Since removal of ethylene from the environment significantly affected both percentage and time taken to abscission (Table 7), the method employed to measure ethylene production in *Impatiens sultani* could not be used here, to obtain consistent results. However, with the same technique it was revealed that explants incubated in the abscission inducing media had a very high rate of ethylene production during the first 12-24 hours of incubation when compared to the controls (Table 8) which were mean rates and standard errors for 5 explants per treatment. The explants in 0.001%

IAA medium exhibited even higher ethylene production rates than explants in 0.0001% IAA media. The basal rate of ethylene production was also higher for explants in 0.001% IAA medium than in others. Some explants in 0.0001% IAA + 0.0001% zeatin medium did show a higher rate of ethylene production just before abscission when they eventually abscised after a significantly longer time than that of controls (cf. Table 7), but in others the difference was not significant.

The method of sealing the explant environment for 2-3 hours to measure the accumulated ethylene produced erratic bursts of ethylene production in some cases which was probably due to the autocatalytic effect of ethylene on its biosynthesis, and did not seem to be the best technique to understand the natural rate of ethylene production during abscission.

3.2.3. Effect of exogenous ethylene on separation layer formation

3.2.3.a. *Impatiens sultani*

Explants cultured in 1.0% sucrose medium (without any hormones in it), could be induced to abscise, by including ethylene into the explant environment. Ethylene concentrations from 5-40 $\mu l\ l^{-1}$ were effective in inducing abscission. However, at higher ethylene concentrations the epidermis of some explants tended to peel off starting from the upper part of the explant. Data in Table 9 are from 2-5 experiments with 8-12 replicates per treatment. Ethylene inclusion immediately after explanting was not effective in promoting abscission, but was effective when ethylene was included 24 hours or 72 hours after explanting (Table 9). Two to three days after ethylene inclusion there was a rapid phase of abscission as illustrated in Fig. 7.

The position of the separation layer was significantly lower (Table 9) in ethylene induced abscission when compared to that of controls ($P<0.02$). Although there is a slight increase in the distance of the separation layer from the base of the explant between explants abscising in response to ethylene application after 72 hours of explanting and that of ethylene applied immediately after explanting (0 hours), more replicates are necessary to demonstrate a statistically different result. The concentration of ethylene used did not significantly affect the position of the separation layer.

Occasionally separation layers formed in response to exogenous ethylene were incomplete in the pith, which was somewhat comparable to adventitious separation layers in *Phaseolus vulgaris* petioles formed in response to IAA and ethylene, which also showed separation layer development restricted to the cortical region (Osborne 1987).

3.2.3.b. *Ipomoea batatas*

Results in table 10 were from 2-3 experiments with 8-12 replicates per treatment. As in *Impatiens sultani* ethylene inclusion into he culture environment 24 hours or 72 hours after explanting induced abscission and inclusion immediately after explanting was not effective (Table 10). The effective ethylene concentrations ranged from 5-20$\mu l\ l^{-1}$. At concentrations higher than this the segments turned yellow or brown very rapidly (within 1-2 days of ethylene application).

The position of the separation layer was again lower than that of the explants in controls without ethylene ($P<0.01$). The position was again not dependent on the concentration of ethylene used or the time of ethylene inclusion after explanting.

4. Discussion

4.1. Involvement of IAA, Zeatin and Ethylene on Separation Layer Development

Results obtained in this study revealed that inclusion of zeatin into the abscission inducing medium in addition to IAA stimulated abscission in both *Impatiens sultani* and *Ipomoea batatas* to a greater extent than with IAA alone.

Ethylene seemed to play a major role in the induction of adventitious abscission with exogenous addition of ethylene into the culture environment inducing abscission in the absence of any other externally applied hormones. However, the frequency of abscission (by including ethylene into the environment) was significantly lower than when abscission was induced by adding IAA (with or without zeatin) into the medium. Including IAA in the medium also resulted in initial high rates of ethylene production by the explant and a peak rate just before abscission.

The initial (within 12-24 hours) high ethylene production by explants was likely to be due to wound ethylene production (Marynick 1977). However, this initial ethylene production was very much greater in explants incubated in media with IAA as compared to that of explants in control medium (with only sucrose). IAA is

known to stimulate ethylene production in many types of tissues (Lau and Yang 1973; Yu *et al.* 1979a; Yang and Hoffman 1984), and carbohydrates, including sucrose had been found to act synergistically with IAA in stimulating ethylene production (Meir *et al.* 1985; Philosoph-Hadas *et al.* 1985). A possible explanation given for this synergism was that carbohydrates may act in maintaining a sufficient level of free IAA (IAA being able to regulate ethylene biosynthesis) to elicit ethylene biosynthesis (Aharoni and Yang 1983; Meir *et al.* 1985). Thus, basally added IAA in addition to contributing towards the setting up of an auxin gradient within the internode, which is thought to be essential for the positional definition of the separation layer (Warren Wilson *et al.* 1986), might also be assisting the process of adventitious abscission, by stimulating ethylene biosynthesis. The inability of *Ipomoea batatas* and *Begonia corallina* explants to be induced to abscise with optimum concentration of IAA but without sucrose (or with low sucrose concentrations) may also be attributed to the above mentioned synergistic effect of carbohydrates on IAA induced ethylene production (sucrose could also be limiting as a respiratory substrate).

The peak rate of ethylene production observed just before abscission was found to be more pronounced when zeatin was included in the culture medium in addition to IAA. The exact relationship between ethylene and cytokinin is still unclear with many reports claiming that cytokinins actually reduce both ethylene synthesis and sensitivity to ethylene (Cook *et al.* 1985; Mor *et al.* 1985; Van Staden *et al.* 1987). As for involvement of cytokinin in abscission some investigators have found that it inhibits abscission (Gorter 1964; Chatterjee and Leopold 1964). Abeles *et al.* (1967)

also reported that cytokinin reduced ethylene sensitivity during abscission. On the other hand, Hanischten Cate and Bruinsma (1973) found cytokinin to produce no inhibition, and Pierik (1980) reported that cytokinin alone could actually accelerate adventitious abscission in pear pedicels. Suttle (1986) provided evidence that cytokinins (including zeatin) are capable of inducing ethylene biosynthesis in non-senescent cotton leaves. The cytokinin, kinetin was also found to act synergistically with IAA in stimulating ethylene production in senescing tobacco leaf discs (Aharoni *et al.* 1979) and *Phaseolus* hypocotyls (Lau and Yang 1973). The stimulation of ethylene production by cytokinin when it occurs is thought to be mediated by its ability to maintain levels of free IAA by preventing conjugation and promoting IAA uptake (Lau and Yang 1973; Lau *et al.* 1977; Hoffman *et al.* 1983). In contrast to Pierik (1980), cytokinins alone could not induce adventitious abscission in the present study. But in combination with IAA, zeatin was able to stimulate abscission with an associated higher ethylene production peak, which could result from the ability of zeatin to enhance ethylene production discussed above. However, at this stage, the faster rate of ethylene production at abscission being an effect of rapid abscission, indirectly caused by zeatin (via an abscission promoting factor other than ethylene) cannot be completely ruled out.

Impatiens sultani and *Ipomoea batatas* internodal explants could also be induced to abscise by exogenous ethylene application which is common to many, normal abscission zones of whole plant and explant systems (see Sexton *et al.* 1985). The exogenous application of ethylene was not effective in inducing abscission when applied immediately after culture but effective if applied 12-24 hours after

explanting. Similar initial insensitivities have also been reported for excised normal abscission zones (Abeles and Rubinstein 1964; de la Fuente and Leopold 1968; Jackson and Osborne 1970; Webster and Leopold 1972). Since ethylene is known to cause inhibition of IAA transport (Beyer and Morgan 1971; Riov and Goren 1979), and possibly conjugation and deactivation (Gaspar *et al.* 1978; Riov *et al.* 1982, 1986), it could be suggested that, application of ethylene immediately after explanting could prevent, or reduce the polar transport of IAA from the upper part of the explant. Thus, preventing setting up of an auxin gradient within the explant which is thought to be important in positional definition of the separation layer during adventitious abscission (Warren Wilson *et al.* 1986). Ethylene applied after 12-24 hours of explanting could be stimulative in inducing abscission, probably via its ability to stimulate the synthesis of hydrolyzing enzymes in the separation layer cells, this being one of the established direct effects of ethylene on abscission (see Sexton *et al.* 1985).

A common approach to implicate ethylene in the process of abscission is to remove endogenous ethylene by absorbing external ethylene (Jackson and Osborne 1970; Young and Meredith 1971). In both *Impatiens sultani* and *Ipomoea batatas* the time taken from explanting to abscission was considerably extended by removing ethylene from the environment. However, unlike *Ipomoea batatas* in *Impatiens sultani* the total percentage of abscission was not significantly lower in the ethylene removed treatments (Table 6). This can be due to the maintenance of sufficiently high levels of ethylene (above threshold concentration) within the tissue even with

ethylene removal from the external environment, at least during the latter stages of separation layer development.

4.2. Separation Layer Position

In *Impatiens sultani* the position of the separation layer was affected by the concentration of basally applied IAA (Warren Wilson *et al.* 1986). *Ipomoea batatas* also showed the same trend, the separation layer being formed at an increasing distance from the base, with increasing concentration of IAA. Therefore, this supports the hypothesis that the separation layer is defined at sites above higher concentrations of auxin caused either by basal accumulation from basipetal transport of auxin or diffusion of auxin from the medium (Warren Wilson *et al.* 1986, 1988). The position of the separation layer in both *Impatiens sultani* and *Ipomoea batatas* was not affected by the application of zeatin into the medium.

When abscission was induced by exogenous application of ethylene it always occurred at a lower position, than when abscission was induced by applying IAA to the basal medium. Its position was also lower than the occasional separation layers that formed in explants in control media, which did not have externally added IAA in the medium. This lowering of the position of the separation layer in response to exogenous ethylene could occur if there was an inhibition of IAA transport or increased deactivation or conjugation of IAA (Beyer and Morgan 1971; Riov *et al.* 1982,1986) within the internode. From the limited number of experiments that were conducted, the time of ethylene application after explanting (0, 24, 72 hours) did not significantly affect the position of the separation layer formed (Table 9-10), which is

probably suggestive that, inhibition of auxin transport was not a major effect of ethylene. However, more experiments are needed to determine its effect.

4.3 Effect of IAA and Zeatin on Rooting of Explants

In both *Impatiens sultani* and *Ipomoea batatas* rooting of explants was stimulated by applying IAA into the medium. However, application of zeatin with IAA (at all concentrations used) inhibited this effect to varying extents. At 0.0001% (4.6×10^{-6} M) zeatin rooting was completely inhibited in both species but at 0.00001% (4.6×10^{-7}M) zeatin its inhibition was not complete. Cytokinins are known to inhibit root formation (Humphries 1960). Jarvis and Yasmin (1987) reported that kinetin at high concentrations (10^{-5}, 10^{-6} M) had an inhibitory effect on rooting of mung bean cuttings when used alone or with IBA (indole buteric acid), but at lower concentrations (10^{-8} M) kinetin tended to act synergistically with IBA in stimulating rooting. Similar experiments using a wider range of concentrations of hormones with *Impatiens sultani* internodal explants (which demonstrated a better capacity to form adventitious roots than *Ipomoea batatas*) should yield interesting results.

Table 1: *Impatiens sultani* ; effect of zeatin concentration (with 1.0% sucrose and with or without 0.0001% IAA), on frequency of abscission and time taken to abscission

Medium		Replicates (no.)	Frequency of Abscission (% ± S.E.)	Time (days±S.E.)
IAA Conc. (%)	Zeatin Conc. (%)			
-	-	20	15.0	17.0±1.2
0.0001	-	72	88.9± 1.9	7.3±0.4
-	0.0001	20	20.0	16.5±1.9
-	0.001	20	10.0	17.5±0.5
0.0001	0.00001	20	85.0	6.3±0.5
0.0001	0.0001	74	97.3± 0.9	5.3±0.2
0.0001	0.001	20	80.0	5.7±0.5

Table 2: *Ipomoea batatas;* effect of different media (with 1.0% or 3.0% sucrose) on frequency of abscission and time taken to abscission

Medium		1.0% sucrose			3.0% sucrose		
IAA Conc. (%)	Zeatin Conc. (%)	Rep (no.)	Freq. of Abscission (% ±S.E.)	Time (days±S.E.)	Rep (no.)	Freq. of Abscission (% ±S.E.)	Time (days±S.E.)
-	-	-	-	-	20	15.0± 5.0	11.3±0.7
0.0001	-	20	70.0± 10.0	11.9± 0.9	40	75.0± 15.6	8.5±0.5
0.001	-	10*	30.0	13.0± 10.0	30	60.0± 5.8	8.0±0.2
0.0001	0.00001	35	97.5± 2.5	11.2± 0.4	20	95.0± 5.0	8.7±0.4
0.0001	0.0001	35	94.0± 4.5	10.1± 0.5	40	95.0± 5.0	7.3±0.3
0.001	0.0001	20	25.0± 5.0	5.8 0.4	10*	50.0	9.1±0.8

Table 3: *Impatiens sultani* ; effect of a range of sucrose concentrations (used with 0.0001% IAA and zeatin) on frequency of abscission and time taken to abscission

Sucrose Conc. (%)	Replicates (no.)	Frequency of Abscission (% ± S.E.)	Time (days ± S.E)
0.00	54	51.0± 16.9	5.5± 0.3
0.03	20	40.0	5.8± 0.7
0.30	20	80.0	6.4± 0.4
1.00	54	96.0± 1.5	5.4± 0.2
3.00	20	95.0	6.0± 0.3

Table 4: *Impatiens sultani*; effect of zeatin on the position of the seperation layer and rooting of explants (based on experiments in table 1)

Medium		Replicates (no.)	Distance from base to seperation layer (mm ± S.E.)	Roots per explant at day 20 (no. ± S.E.)
IAA Conc. (%)	Zeatin Conc. (%)			
-	-	20	4.8± 0.4	0.8±0.3
0.0001	-	72	6.9± 0.2	4.3±0.5
-	0.0001	20	5.6± 0.8	0.0
-	0.001	20	5.5± 0.5	0.0
0.0001	0.00001	20	6.8± 0.3	0.6±0.3
0.0001	0.0001	74	6.7± 0.1	0.0
0.0001	0.001	20	7.0± 0.3	0.0

Table 5: *Ipomoea batatas* ; effect of different media (with 3.0% sucrose) on the position of the seperation layer and root formation (based on experiments in Table 2)

Medium		Replicates	Distance from base to seperation layer	Roots per explant at day 20
		(no.)	(mm ± S.E.)	(no.± S.E.)
IAA Conc. (%)	Zeatin Conc. (%)			
-	-	20	4.5± 0.3	0.3± 0.1
0.0001	-	40	5.0± 0.2	1.3± 0.2
0.001	-	30	6.8± 0.5	4.5± 0.5
0.0001	0.00001	20	5.1± 0.5	0.4± 0.1
0.0001	0.0001	40	4.9± 0.2	0.0
0.001	0.0001	10	7.0± 0.5	0.0

Table 6: *Impatiens sultani* ; effect of removing ethylene from environment on frequency of abscission and time taken to abscission of explants, cultured in two abscission inducing media (0.0001% IAA + 1.0% sucrose and 0.0001% IAA + 0.0001% zeatin + 1.0% sucrose) and control medium (1.0% sucrose). (R) = ethylene removed , (NR) = ethylene not removed (control)

Medium		Treatment	Replicates (no.)	Frequency of Abscission (% ± S.E.)	Time (days ± S.E.)
IAA Conc. (%)	Zeatin Conc. (%)				
-	-	R	16	0.0	-
,,	,,	NR	16	12.5± 12.5	12.0±2.0
0.0001	-	R	24	67.5± 7.5	9.2±0.9
,,	,,	NR	20	75.0± 5.0	7.9±0.6
0.0001	0.0001	R	24	84.4± 9.4	8.5±0.6
,,	,,	NR	20	95.0± 5.0	6.2±0.6

Table 7: *Ipomoea batatas* ; effect of removing ethylene from environment on frequency of abscission and time taken to abscission of explants cultured in two abscission inducing media (0.0001% IAA + 3.0% sucrose and 0.0001% IAA + 0.0001% zeatin + 3.0% sucrose) and control medium (3.0% sucrose). (R) = ethylene removed , (NR) = ethylene not removed (control)

Medium		Treatment	Replicates (no.)	Frequency of Abscission (% ± S.E.)	Time (days ± S.E.)
IAA Conc. (%)	Zeatin Conc. (%)				
-	-	R	16	6.3± 5.9	9.0
-	-	NR	10	10.0	11.0
0.0001	-	R	20	35.0± 5.0	11.2±1.1
„	„	NR	20	70.0± 9.0	8.7±0.5
0.001	-	R	18	67.5± 7.5	7.8±0.5
„	„	NR	20	65.0± 5.0	7.7±0.3
0.0001	0.0001	R	20	65.0± 5.0	10.3±0.8
„	„	NR	20	95.0± 5.0	7.8±0.4

Table 8: *Ipomoea batatas* ; rate of ethylene evolution at different time intervals after culture in three abscission inducing media and control medium

Medium		Rate of ethylene production ($nl.h^{-1}g.^{-1}f.wt. \pm S.E.$)				
IAA Conc. (%)	Zeatin Conc (%)	8 h	18 h	42 h	66 h	90 h
-	-	1.1±0.3	0.5±0.3	0.2±0.2	0.1±0.1	0.1±0.9
0.0001	-	2.8±0.5	1.6±0.3	0.9±0.2	0.3±0.2	0.3±0.3
0.001	-	8.5±1.2	4.5±1.7	2.1±0.4	1.9±0.4	2.0±0.4
0.0001	0.0001	6.0±1.4	3.3±0.8	1.3±0.3	0.4±0.2	0.5±0.3

Table 9: *Impatiens sultani* ; effect of introducing ethylene (10 µl/l), into the environment of the explant at different time intervals after explanting (in 1.0 % sucrose) on abscission

Time of ethylene inclusion (h)	Replicates (no.)	Frequency of Abscission (% ± S.E.)	Time (days± S.E)	Distance to seperation layer from base (mm ± S.E.)
0	19	15.6±4.5	6.0±2.1	3.3±0.2
24	56	66.7±6.2	4.6±0.2	3.5±0.1
72	16	56.3±6.3	7.3±0.8	3.8±0.1
Control	28	17.5±6.3	12.4±1.6	4.6±0.2

Table 10: *Ipomoea batatas* ; effect of introducing ethylene (10 µl/l),into the environment of the explant at different time intervals after explanting (in 3.0 % sucrose) on abscission

Time of ethylene inclusion (h)	Replicates (no.)	Frequency of Abscission (% ±S.E.)	Time (days±S.E)	Distance to seperation layer from base (mm ±S.E.)
0	16	6.3±6.3	4.0	3.5
24	30	66.6±6.7	5.1±0.5	3.8±0.1
72	24	62.5±7.2	6.8±0.4	3.9±0.1
Control	24	16.7±9.5	10.8±1.3	5.0±0.4

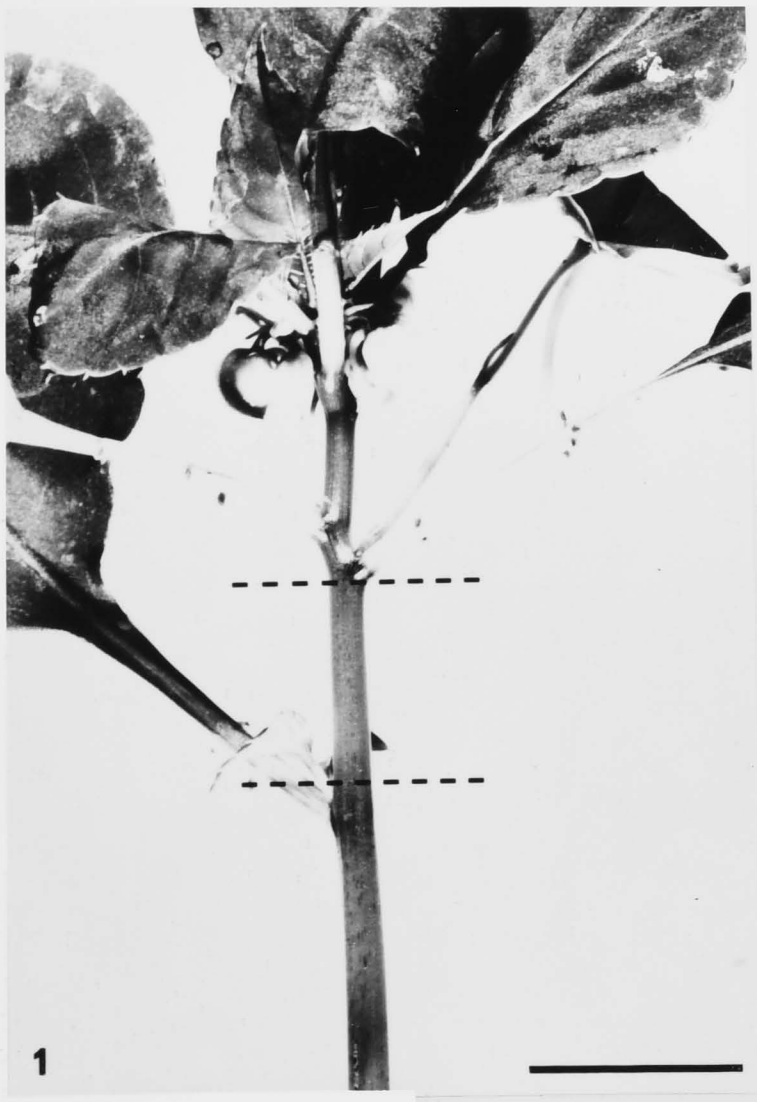
Figs 1-3. Photographs illustrating shoots used to obtain internodal explants for culture. The typical internodes used are denoted by the broken lines.

Scale bar = 20mm

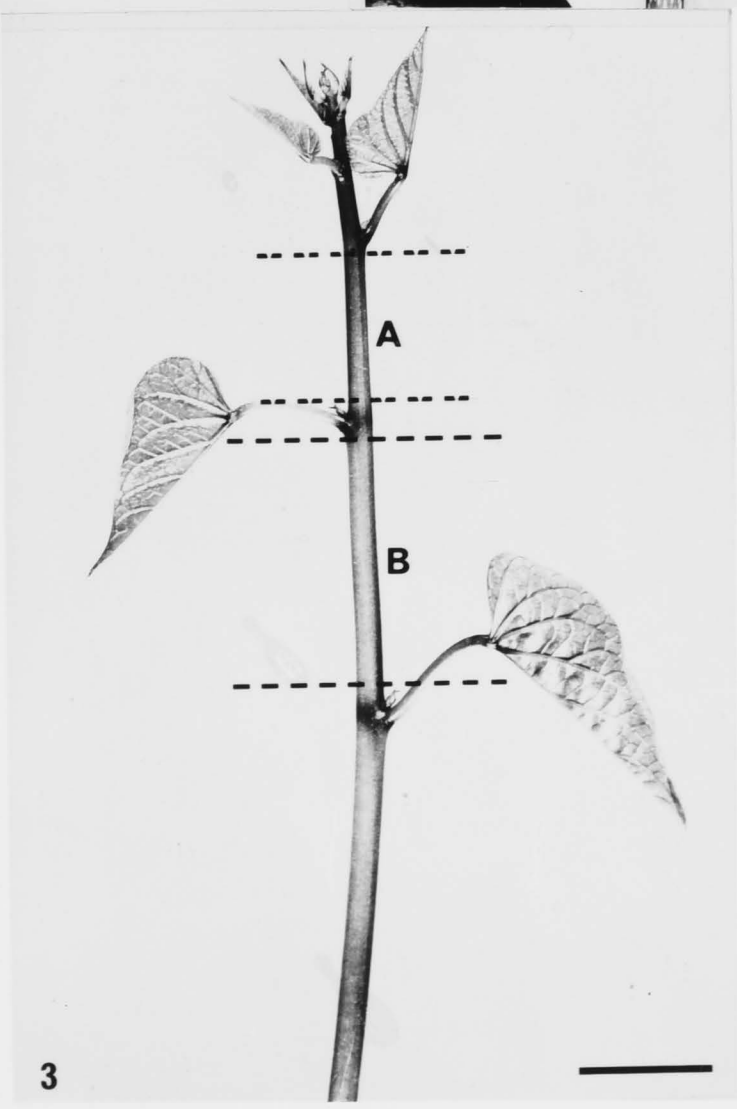
Fig. 1. *Impatiens sultani*

Fig. 2. *Begonia corallina*

Fig. 3. *Ipomoea batatas*. A = internode used to obtain the least mature explant (referred to in the text as very young internodal explant, Chapter 2); B = internode used to obtain the more mature explant (referred to as young internodal explant, Chapters 1 and 2).


1


2

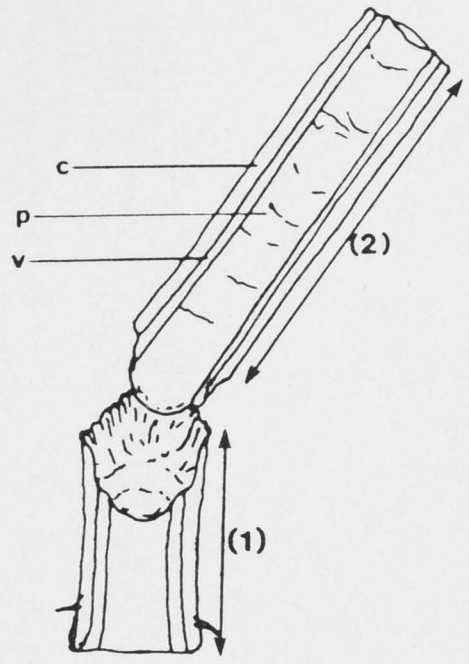


3

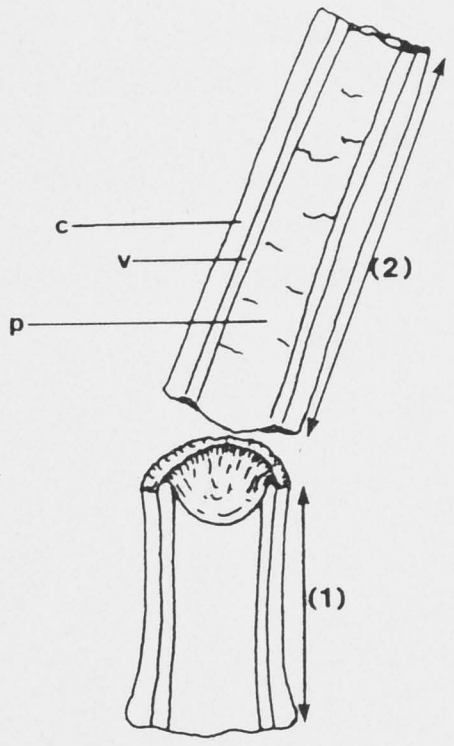
Fig. 4. Illustrations of *Impatiens sultani*, *Ipomoea batatas* and *Begonia corallina* internodal explants at abscission (halved longitudinally), showing the form of the separation layer region and distances measured.

(1) = distance from base to the separation layer

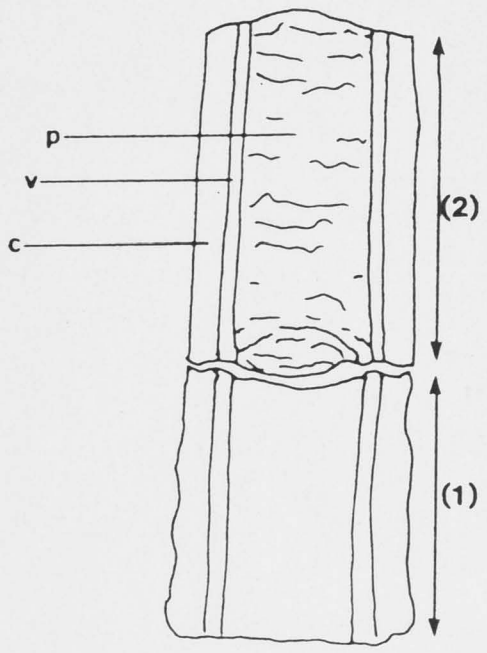
(2) = distance from upper end to the separation layer p= pith; v = vascular region; c = cortex.



Impatiens sultani



Ipomoea batatas



Begonia corallina

Fig. 4 10mm

Fig. 5. Progress of abscission (expressed as a percentage) in *Impatiens sultani* explants cultured in two abscission inducing media (0.0001% IAA + 1.0% sucrose and 0.0001% IAA + 0.0001% zeatin + 1.0% sucrose) and in the control medium (1.0% sucrose). The values are means of 5 experiments and their standard errors.

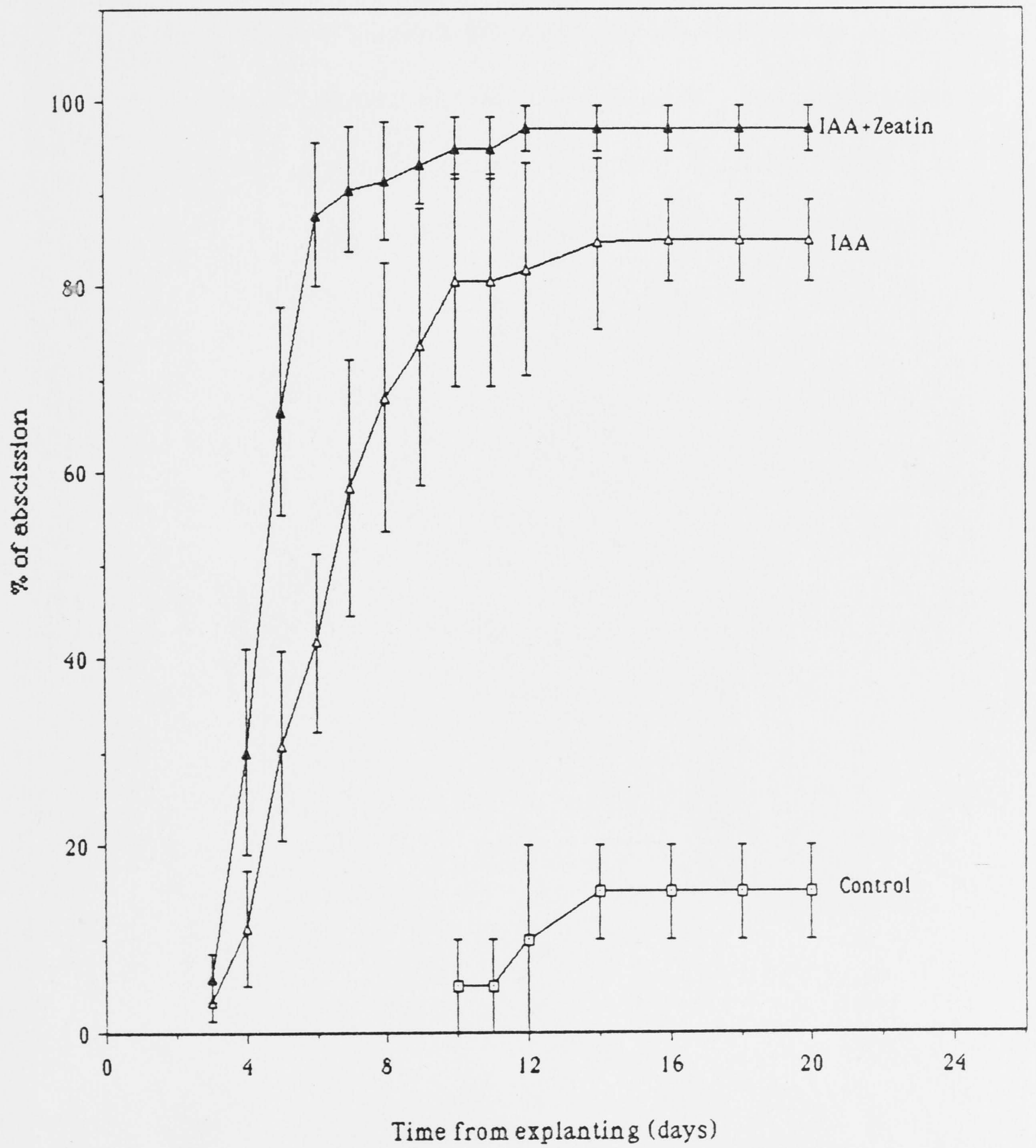

100
80
60
40
20
0
% of abscission
IAA+Zeatin
IAA
Control
0
4
8
12
16
20
24
Time from explanting (days)

Fig. 5

Fig. 6. The rate of ethylene production during incubation, by *Impatiens sultani* explants in two abscission inducing media (0.0001% IAA + 1.0% sucrose and 0.0001% IAA + 0.0001% zeatin + 1.0% sucrose) and the control medium (1.0% sucrose). The results presented are means and standard errors of 6 measurements. Arrows indicate the time of explant abscission in the two abscission inducing media.

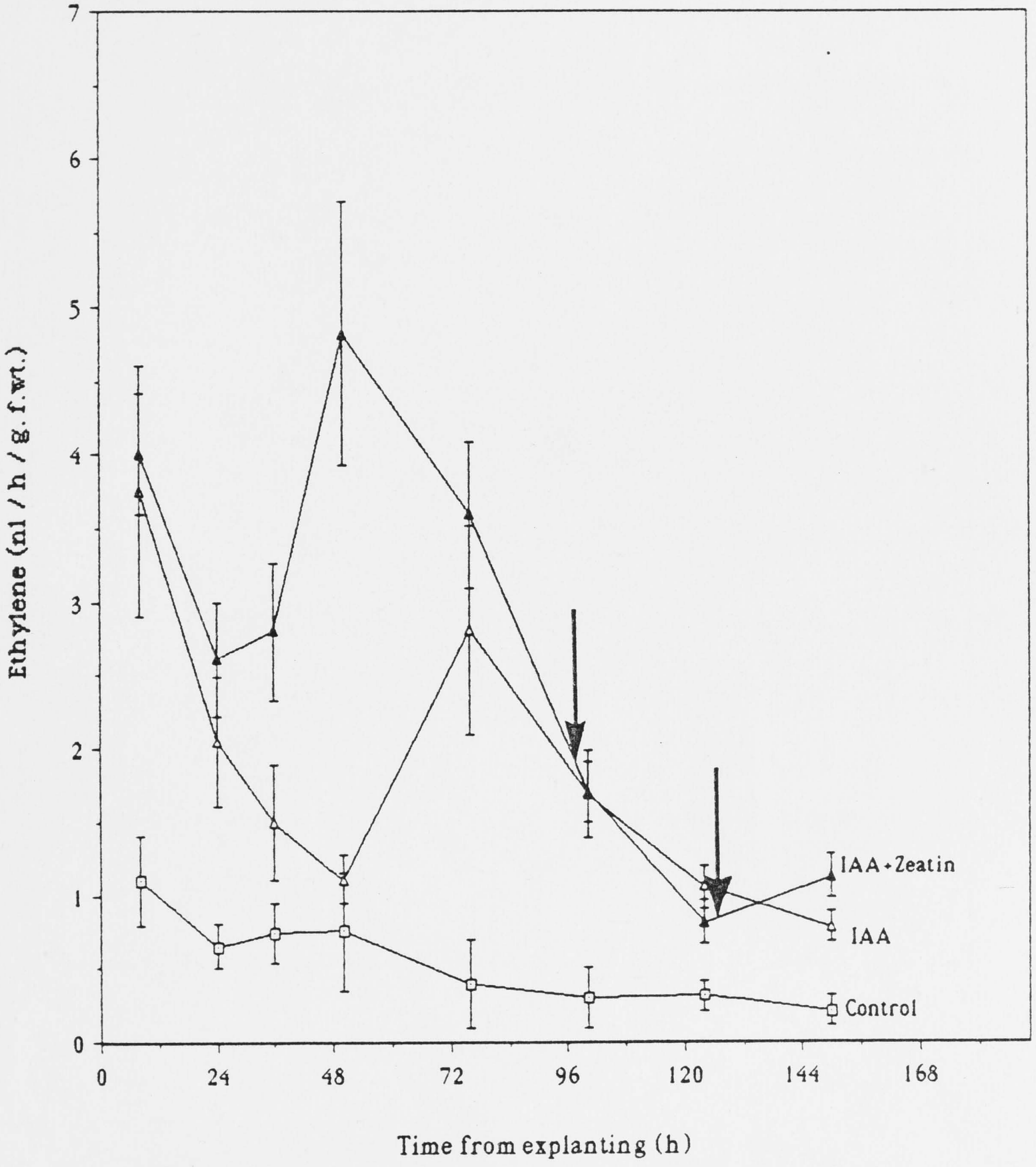

Ethylene (nl / h / g. f.wt.)
7
6
5
4
3
2
1
0
0
24
48
72
96
120
144
168
IAA + Zeatin
IAA
Control
Time from explanting (h)

Fig. 6

Fig. 7. Progress of abscission *Impatiens sultani* (expressed as a percentage of abscission) when 10 µl l^{-1} of ethylene is included into the environment, 24 hours after explanting (and control without ethylene inclusion) of explants, cultured in sucrose (1.0%) only media. The results are means±SE of 5 experiments.

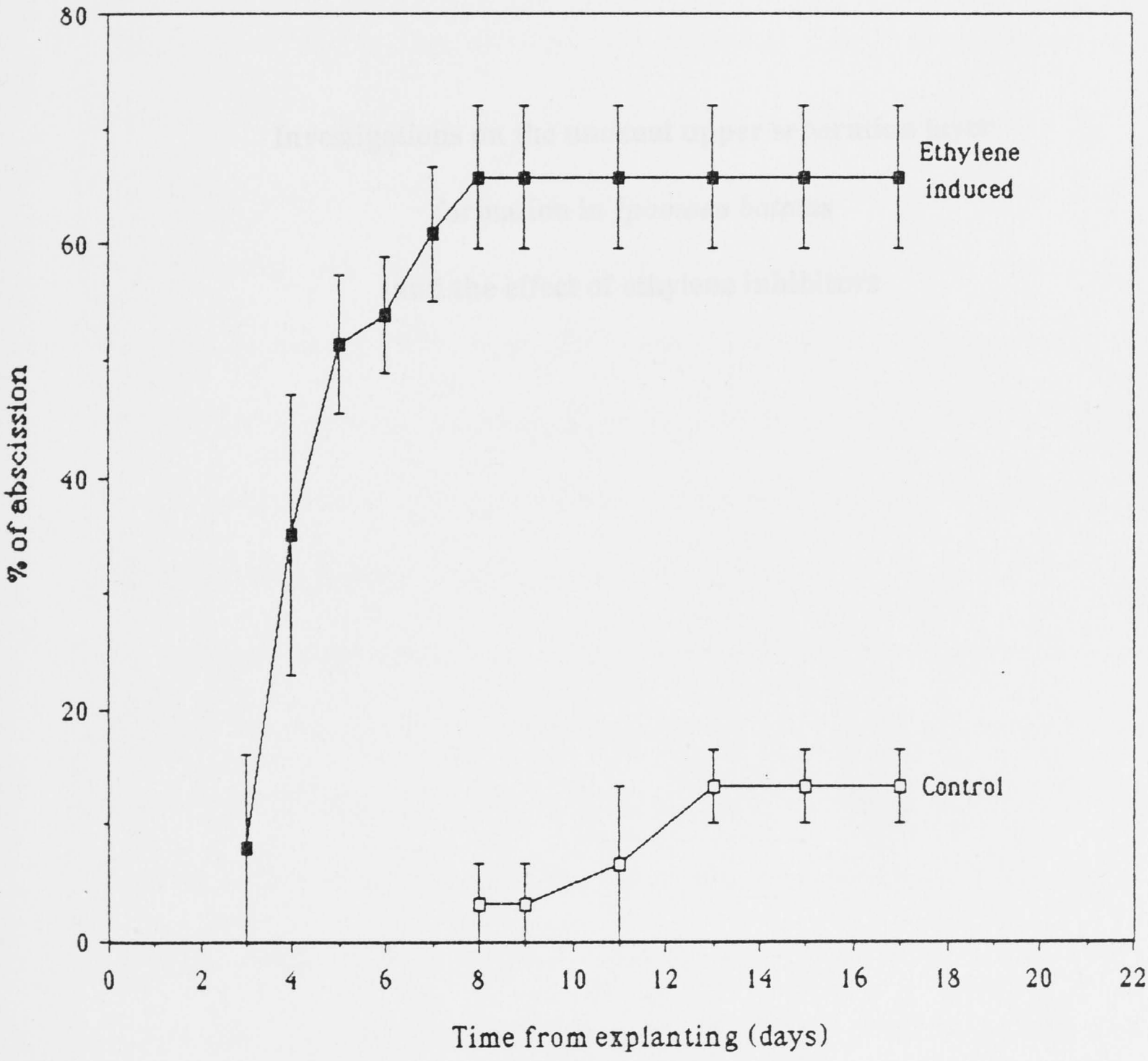

80
60
40
20
0
% of abscission
Ethylene
induced
Control
0
2
4
6
8
10
12
14
16
18
20
22
Time from explanting (days)

Fig. 7

CHAPTER TWO

Investigations on the unusual upper separation layer formation in *Ipomoea batatas* and the effect of ethylene inhibitors

1. INTRODUCTON

Ipomoea batatas internodal explants when cultured in abscission inducing media, occasionally develop unusual upper separation layers a few millimeters below the upper exposed surface, in addition to the lower separation layer (Chapter 1). This upper separation layer develops soon after explanting, prior to the lower separation layer. Chapter 1 noted a higher rate of ethylene production by explants cultured in abscission inducing media, just after explanting (but not in explants cultured in control media), which was attributed to wound ethylene production. Interestingly, the time of upper separation layer development was found to coincide with this high ethylene production. Ethylene being a potent promoter of abscission (see Chapter 1), it could be argued that this upper separation layer is formed by a wound response in association with initial high ethylene production.

Amino-ethoxyvinylglycine (AVG) being an inhibitor of enzyme ACC synthase (which is the crucial enzyme for the conversion of SAM → ACC during ethylene biosynthesis, Yu *et al.* 1979b; Yang 1987) has been used to demonstrate ethylene involvement in normal abscission in a variety of plants (Bengerth 1978; Sagee *et al.* 1980; Devenport and Manners 1982; Sipes and Einset 1982; Roberts *et al.* 1984). Similarly Ag^{+} and anionic sliver thiosulphate (STS) has been used to block ethylene action (Beyer 1976) in abscission experiments (Sagee *et al.* 1980; Cameron and Reid 1981; Curtis 1981; Kushad and Pooaviah 1984).

The present investigation attempts to understand the process of abnormal upper separation layer development in *Ipomoea batatas* and involvement of ethylene in both upper and lower separation layer formation, by the use of ethylene biosynthesis

inhibitor AVG and ethylene antagonist STS. Since the separation layer forming ability can change with the maturity of the explant (Warren Wilson *et al.* 1986), explants at two maturities were compared in most of the experiments. As the abscission inducing media, it was decided to use 0.001% IAA medium in addition to the standard 0.0001%IAA ± 0.0001% zeatin media, since high IAA medium was observed to stimulate more ethylene production in explants than the low IAA medium (Chapter 1).

2. MATERIALS AND METHODS

2.1. Selection of Plant Material

Plants of *Ipomoea batatas* were grown as described in Chapter 1. Only the actively elongating shoots with flexible long internodes were used for obtaining explants. From these shoots, the youngest internode measuring more than 20mm and less than 40mm (usually the internode below the 2^{nd} or 3^{rd} recognizable node from the apex), was used to obtain the least mature explants (referred as very young internodal explants). The internode below this yielded the more mature explant (referred as young internodal explants), which was the standard explant used for all the previous experiments covered in Chapter 1 (Chapter 1, Fig. 3). When internodes were selected for very young internodal explants it was ensured that the internode below this was suitable for obtaining young internodal explants.

2.2. Culture

Explant preparation, media preparation, culture procedure and recording of results were as described in Chapter 1. The standard errors of percentages of abscission were derived from values for each experiment, while the standard errors of time taken to abscission and the distance to separation layer from the base were derived from values for individual explants.

2.3. Treatment With AVG and STS

AVG, and STS (prepared by mixing equal volumes of $AgNO_3$ and $Na_2S_2O_3.5H_2O$ in sterile water in a molar concentration ratio of 1:8, respectively, Veen 1979) at appropriate concentrations were prepared in sterile distilled water. Apical application of AVG and STS to explants were made as two, 2µl droplets (with 10 minute time intervals in between), immediately after culture. For controls a similar application of deionized water was used. The inclusion of AVG and STS (at appropriate concentrations) into the media were done just before pouring the autoclaved culture media into the culture vials.

When required lanolin application to the upper cut surface was carried out immediately after culture, with hydrous lanolin using a hypodermic syringe.

3. RESULTS

The upper separation layer formed 0.5-5.0 mm below the upper end of the explant, completing 3-4 days after explanting. It always preceded the normal lower separation layer. Rarely when the lower separation layer formed fast the upper

separation layer did not get completely established after the initiation and instead became indistinguishable after a few days.

The course of this upper separation layer across the stem was very much similar to the lower separation layer (Chapter 3), although occasionally it tends to spread diagonally across the stem (Chapter 3, Figs 6-7). The characteristic yellowing of the region above the future separation layer was common to both these separation layers. The development of these two separation layers occurred independent from each other and sometimes explants produced only the upper separation layer but not the lower separation layer. Though the lower separation layers occasionally formed in control media without IAA, the upper separation layers were never observed in explants in control media. However, inclusion of exogenous ethylene to the environment of the explants cultured in control media was able to induce upper separation layer formation.

3.1. Effect of Internodal Maturity

Results in Tables 1 and 2 are from 2-5 experiments with 7-12 replicates per treatment. These data show that in all three media, the explants obtained from very young internodes had a better capacity to form upper separation layers than explants from normally used young internodes. However, the time taken for upper separation layer completion did not differ significantly, either between very young, and young internodal explants or between the type of medium used for inducing abscission. Explants in 0.01% IAA medium produced a significantly greater percentage of upper separation layers than explants in media with lower IAA concentration (0.0001%), in

very young internodal explants ($P<0.05$ with 0.0001% IAA and 0.0001% IAA + 0.0001% zeatin media). This increase in percentage of upper separation layers in response to high IAA was not that significant in young internodal explants ($P<0.1$). Addition of zeatin to 0.0001% IAA media demonstrated a slight increase in the percentage of upper separation layers, in both young and very young internodal explants, which was not statistically significant ($P>0.5$).

The lower separation layer took a longer time to complete in very young internodal explants than in young internodal explants ($P<0.01$), except in 0.0001% IAA + 0.0001% zeatin medium which did not show a statistically significant difference ($P>0.4$). The percentage of the lower separation layers formed, however, did not change significantly with the maturity except in the medium with 0.0001% IAA, which showed a markedly high percentage of abscission in young internodal explants than in very young internodal explants ($P<0.05$).

3.2. Effect of Covering the Upper Exposed Surface with Lanolin

Covering the exposed cut surface of the explant with hydrous lanolin significantly reduced the occurrence of the upper separation layer. Table 3 presents the average results of 2-3 experiments (with 8-10 replicates per treatment). Explants cultured in all 3 media exhibited a reduction in upper separation layer formation in response to lanolin application ($P<0.01$, $P<0.02$, $P<0.05$ for 0.0001% IAA, 0.0001% IAA + 0.0001% zeatin and 0.001% IAA media respectively). The time taken for the completion of the separation layer, however, remained roughly the same irrespective

of lanolin cover or the type of medium used. Lanolin cover did not significantly affect the normal lower separation layer formation (results not tabulated).

3.3. Effect of AVG

Data in table 4 were from 2-3 experiments with 5-12 replicates per treatment. AVG when applied to the basal medium at 0.1 mM concentration was very effective in reducing the occurrence of both the upper and the lower separation layer in explants obtained from very young internodes. However, in young explants the lower separation layer was suppressed only to a lesser extent.

When 0.2 mM AVG is applied as two 2 µl droplets on to the upper cut surface of the explant, the upper separation layer formation was inhibited in the 2 media with low IAA concentration (0.0001%), but in the medium with a high IAA concentration (0.001%), the inhibition was not marked. Even doubling the AVG concentration (0.4mM) did not significantly affect the ineffectiveness on inhibition. Its inhibitive effect on the lower separation layer when applied apically was only marked in 0.0001% IAA medium. Again in all 3 media the young internodal explants exhibited a lower inhibition of lower separation layer than the very young internodal explants.

3.4. Effect of STS

Table 5 presents data from 1 or 2 experiments with 6-10 replicates per treatment, done parallel with experiments using AVG (Table 4). When STS was applied apically, it inhibited the upper separation layer formation almost completely,

in both very young and young internodal explants, in all 3 media. The lower separation layer formation was also inhibited to a considerable extent.

However, the basal application of STS was not effective in inhibiting the upper separation layer in all 3 media. The lower separation layer on the other hand, was inhibited to a considerable extent in both young and very young internodal explants.

3.5. Effect of AVG and STS on the Position of the Separation Layer

The position of the upper separation layer was inconsistent (varying between 0.5-5.0 mm) even in the controls, and apical or basal application of AVG or STS did not produce a discernible effect on the position of the upper separation layers (if they formed).

Apical application of AVG and STS did not affect the position of the lower separation layer in any of the 3 media used when compared to their respective controls. In contrast the limited number of explants That abscised, with basal application of AVG and STS, developed the separation layer further away from the base of the explant, in all 3 media, in both young and very young internodal explants (Table 6, data presented are from experiments described in Table 4 and 5). Basal application of STS was the most effective in increasing the distance of the separation layer from the base of the explant.

4. DISCUSSION

4.1. Upper Separation Layer

AVG, being an inhibitor of enzyme ACC synthase (Boller *et al.* 1979; Yang 1987) which is involved in the conversion of SAM → ACC during ethylene biosynthesis, was able to inhibit the upper separation layer formation when applied apically (although it is not effective to the same extent as STS). This inhibitory effect was counteracted by the use of a higher concentration of IAA (0.001% IAA) in the medium. However, when AVG was applied basally it very effectively inhibited the upper separation layer formation in explants, in all media including the high IAA (0.001% IAA) medium. IAA is known to enhance ethylene production via its stimulatory effect on the synthesis of ACC synthase which converts SAM → ACC (Yoshii and Imaseki 1981) and basal part of the explants being in contact with the IAA in the medium may be more involved in the production of ethylene. Explants in the high IAA medium was found to produce more ethylene than explants in low IAA media (Chapter 1). Thus the ineffectiveness of upper separation layer inhibition by apically applied AVG in explants in high IAA medium could be due to the supplementation of ethylene deficiency of the upper parts (due to inhibition of synthesis by AVG), by IAA stimulated ethylene synthesis by the basal parts of the explants.

STS when applied apically, inhibits upper separation layer formation in explants obtained from very young as well as young internodes. Ag^{+} is known to be a potent antagonist of ethylene action (Beyer 1976) and is thought to interact with the ethylene binding sites, thus making it non-responsive to ethylene (Yang 1985). The complete inhibition of upper separation layer by apically applied STS strongly indicate that ethylene is actively involved in the upper separation layer development.

Interestingly, when ethylene was applied basally it was not effective in preventing the upper separation layer development in contrast to AVG. Ag^+ being only and antagonist of ethylene action does not inhibits its synthesis. (Lis *et al.* 1984), and it is also known to stimulate ethylene production especially in combination with IAA or IAA and kinetin in aging leaf discs (Aharoni *et al.* 1979). If Ag^+ fails to reach the upper parts of the explant in time (when applied basally), it will not be able to inhibit the upper separation layer since ethylene produced by the explant in response to IAA and wounding would be able to act on forming the upper separation layer, which developed relatively faster than the lower separation layer.

The actual initiation of the upper separation layer should results from the creation of a localized auxin gradient, at the upper end of the explant and is likely to be caused by a wound response. Wounding is known to accelerate IAA breakdown or oxidation at the exposed surface (Briggs *et al.* 1955; Ray 1958; Zenk and Muller 1964), which could account for the creation of a localized low auxin region close to the upper wound surface and a higher auxin region below it. Preliminary attempts to modify and increase the wound area by cutting the explant end diagonally did not produce any conclusive results. Covering the wound surface with lanolin which could reduce the oxidation of IAA at the wound surface did, however, reduce the occurrence of the upper separation layer. Covering the wound area with lanolin could also reduce the amount of wound ethylene synthesized since ethylene forming enzyme (enzyme which converts ACC $\rightarrow$ ethylene) is dependent on O_2. Anaerobiosis has been reported to stop wound ethylene synthesis (Salteveit and Dilley 1978, 1979).

Ethylene liberated at the wound area after explanting could also be involved in localized deactivation or conjugation of IAA at the wound area, thus, assisting in the creation of an auxin gradient.

The inconsistent position and occasional irregular shape of the upper separation layer could also suggest that its development is dependent on the extent or nature of wounding during explant preparation, which in turn affect the subsequent wound associated changes discussed above.

Ethylene is essentially involved in the upper separation layer development either in assisting the creation of a localized auxin gradient close to the wound area or by acting on already defined separation layer (target) cells, since:

(1) Unlike lower separation layers, upper separation layers were never observed in explants cultured in control media without any hormones, which produced very little ethylene (Chapter 1).

(2) Upper separation layers could be induced by adding exogenous ethylene into explants, cultured in control media.

(3) More upper separation layers were formed in media with high IAA, which liberated more ethylene than explants in low IAA media.

(4) Ethylene antagonist STS completely inhibited upper separation layer development, when applied apically.

(5) Ethylene biosynthesis inhibitor AVG was more effective when applied basally to explants in both high IAA or low IAA media, than when applied apically, which presumably prevents the IAA stimulated ethylene biosynthesis at the basal region.

On these grounds it could be argued that the higher frequency of upper separation layer occurrence, in explants obtained from very young internodes, than from young internodes is possibly due to their higher capacity to produce ethylene. However, a significant difference in the ethylene production between very young and young internodal explants could not be detected. The difference in the capacity to form upper separation layers, with maturity of the explant may reflect differences in the physiological condition, such as differences in the auxin status, transport or metabolism which would differ depending on the maturity. It could also be said that the less mature internodal cells are more flexible and could have a better potential to become committed as separation layer cells when the appropriate inductive conditions are present. However, the capacity to form the lower separation layer in very young internodal explants was actually lower than that of young internodal explants (Table 1-2).

4.2. Lower Separation Layer

Both AVG and STS were able to inhibit the lower separation layer to some extent, when applied apically or basally. As in the upper separation layer, AVG was less effective in inhibiting lower separation layer when applied apically to explants in high IAA media and also to explants in low IAA + zeatin media. This again could be attributed to zeatin (discussed in Chapter 1) or high IAA concentration's ability to stimulate ethylene production, by the basal part of the explant which could restrain AVG's inhibitory effect on ethylene synthesis in the upper part of the explant.

Results in the Table 5 show that, unlike the upper separation layer, the lower separation layer was inhibited to somewhat similar extent, irrespective of basal or apical modes of STS application in all 3 media. STS was effective in inhibiting the lower separation layer even when applied apically to explants in high IAA medium, which seems to suggest that apically applied STS is able to move to the lower parts of the explant in time to prevent ethylene action and thus the lower separation layer development (lower separation layer typically requiring a longer time to develop than the upper separation layer).

It is clear from results in Tables 1 and 2 that very young internodal explants took a longer time to abscission, than young internodal explants in all three media, although the total percentage of abscission was not significantly different (except in very young internodal explants in 0.0001% IAA medium, which had a significantly lower percentage of abscission). This difference between very young and young internodal explants was also demonstrated when AVG is applied basally, where very young internodal explants were inhibited to a significantly greater extent. This difference between maturity of explants should reflect some variation in the physiological condition between them. However, it should be noted that very young internodal explants had a better capacity to form the upper separation layer, unlike the lower separation layer. Which seems to suggest that the inductive conditions required for upper and lower separation layers are some what different.

The change in the position of the separation layer (forming further away from the base), in response to basal AVG or STS treatment is thought provoking. It is likely to caused by inhibition of some ethylene action such as IAA metabolism or

transport, which in turn would create a higher auxin level in the basal parts of the explants, than in non-treated explants and further experiments on this aspect should yield interesting results.

Table 1: Effect of three abscission inducing media on frequency and time taken to abscission of **very young** internodal explants. U.S.L. = upper separation layer , L.S.L. = lower separation layer

Medium		Replicates (no.)	U.S.L.		L.S.L.	
IAA Conc (%)	Zeatin Conc (%)		Frequency (% ± S.E.)	Time (days±S.E.)	Frequency (% ± S.E.)	Time (days±S.E.)
0.001	-	22	100.0	3.7±0.1	81.8±2.1	12.3±0.5
0.0001	-	24	76.0± 10.2	3.8±0.1	45.0±9.9	11.4±1.2
0.0001	0.0001	34	84.7± 9.9	3.9±0.1	97.2±4.0	8.0±0.5

Table 2: Effect of three abscission inducing media on frequency and time taken to abscission of **young** internodal explants. U.S.L. = upper separation layer , L.S.L. = lower separation layer

Medium		Replicates (no.)	U.S.L.		L.S.L.	
IAA Conc (%)	Zeatin Conc (%)		Frequency (% ± S.E.)	Time (days±S.E.)	Frequency (% ± S.E.)	Time (days±S.E.)
0.001	-	24	58.3± 9.8	3.7±0.2	78.2±6.5	8.0±1.0
0.0001	-	38	25.6± 11.0	3.8±0.3	89.5±5.8	7.7±0.5
0.0001	0.0001	54	35.2± 4.9	3.9±0.1	98.0±2.0	7.5±0.3

Table 3: Effect of covering the upper exposed surface of very young internodal explants with lanolin on frequency and time taken for upper seperation layer formation.
(+) = lanolin covered, (-) = control without lanolin cover

Medium		Lanolin cover	Replicates (no.)	Frequency of abscission (% ± S.E.)	Time (days±S.E.)
IAA Conc. (%)	Zeatin Conc. (%)				
0.0001	-	(+)	24	16.7± 12.1	3.6±0.3
,,	,,	(-)	30	60.0± 5.8	3.8±0.2
0.001	-	(+)	18	40.0± 10.0	3.6±0.2
,,	,,	(-)	20	95.0± 5.0	3.3±0.1
0.0001	0.0001	(+)	18	26.1± 3.9	3.4±0.3
,,	,,	(-)	20	95.0± 5.0	3.4±0.1

Table 4; Effect of applying 0.2mM AVG apically (as two 2µl droplets) or 0.1mM in the basal medium on frequency of upper seperation layer (U.S.L.) and lower seperation layer (L.S.L.) formation , in explants cultured in three abscission inducing media. V.Y = very young internodal explants, Y = young internodal explants, Bas. = AVG basally, Apic. = AVG apically, Control =deionized water apically

Medium		AVG Application	Maturity	Rep. (no.)	Frequency U.S.L. (% ± S.E.)	Frequency L.S.L. (% ± S.E.)
IAA Conc. (%)	Zeatin Conc (%)					
0.0001	-	Bas.	V.Y	14	14.6± 3.0	14.6± 3.0
,,	,,	,,	Y.	10	10.0	50.0
,,	,,	Apic.	V.Y	14	35.7	14.3
,,	,,	,,	Y.	10	10.0	40.0
,,	,,	Control	V.Y.	10	70.0	60.0
,,	,,	,,	Y.	20	25.0± 5.0	80.0± 9.9
0.001	-	Bas.	V.Y	21	14.1± 5.8	14.6± 7.7
,,	,,	,,	Y.	18	11.3± 1.8	56.3± 8.8
,,	,,	Apic.	V.Y	26	80.8± 9.0	50.0± 7.0
,,	,,	,,	Y.	22	27.5± 3.5	67.5± 6.5
		Control	V.Y.	18	95.0± 5.0	78.8± 8.8
,,	,,	,,	Y.	20	50.0± 10.0	80.0± 10.0
0.0001	0.0001	Bas..	V.Y	18	0.0	11.3± 1.8
,,	,,	,,	Y.	12	8.3	50.0
,,	,,	Apic.	V.Y	24	20.8± 8.3	62.5± 7.2
,,	,,	,,	Y.	19	22.6± 8.4	89.5± 11.8
,,	,,	Control	V.Y.	15	88.0	97.0
,,	,,	,,	Y.	20	35.0± 5.0	100.0

Table 5: Effect of applying 0.1mM STS apically (as two 2µl droplets) or in the basal medium on seperation layer formation (for controls refer Table 4)

Medium		STS Application	Maturity	Rep. (no.)	Frequency U.S.L. (% ± S.E.)	Frequency L.S.L. (% ± S.E.)
IAA Conc. (%)	Zeatin Conc (%)					
0.0001	-	Apic.	V.Y	10	0.0	10.0
,,	,,	,,	Y	9	0.0	22.2
,,	,,	Bas.	V.Y	12	91.7	33.3
,,	,,	,,	Y	10	40.0	20.0
0.001	-	Apic.	V.Y	16	0.0	18.8± 8.8
,,	,,	,,	Y	10	0.0	30.0
,,	,,	Bas.	V.Y	15	66.7	26.7
,,	,,	,,	Y	16	25.0	31.3
0.0001	0.0001	Apic.	V.Y	20	0.0	40.0± 14.1
,,	,,	,,	Y	10	10.0	20.0
,,	,,	Bas..	V.Y	19	68.9± 12.6	37.2± 10.2
,,	,,	,,	Y	10	20.0	40.0

Table 6: Effect basal application of AVG (0.1mM) and STS (0.1mM) into the medium on position of the lower seperation layer (results presented are from the experiments described in Table 4 and 5)

Medium		Inhibitor	Distance to seperation from base (mm ±S.E.)	
IAA Conc. (%)	Zeatin Conc. (%)		Very young	Young
0.0001	-	-	4.8±0.4	5.0±0.3
„	„	AVG	5.5±0.5	6.3±0.9
„	„	STS	6.0±1.7	6.3±1.1
0.001	-	-	6.4±0.4	6.8±0.3
„	„	AVG	8.0±0.4	8.1±0.8
„	„	STS	10.5±0.6	11.1±1.1
0.0001	0.0001	-	4.8±0.3	4.9±0.2
„	„	AVG	5.5±0.5	5.1±0.3
„	„	STS	8.6±0.8	7.6±1.0

CHAPTER THREE

Anatomical and ultrastructural aspects of adventitious abscission in internodal explants of *Impatiens sultani*, *Ipomoea batatas*, and *Begonia corallina*.

1. INTRODUCTION

Anatomical and ultrastructural changes associated with normal (primary) abscission have been studied extensively (Valdovinos *et al.* 1968; Sexton and Hall 1974; Sexton 1976; Gilliland and Van Staden 1985). These investigators have reported that in most cases abscission is achieved by cell separation due to the secretion of hydrolytic enzymes by separation layer cells upon their activation. Dictyosomes are implicated in this enzyme secretory process (Sexton *et al.* 1977; Sexton and Roberts 1982; Osborne and McManus 1986).

Similar studies on adventitious (secondary) abscission have been limited. In such investigations Webster and Leopold (1972) have reported cell breakdown and cell division during adventitious abscission of *Phaseolus vulgaris* stems, later Osborne and McManus (1986) reported that adventitious abscission observed in *Phaseolus vulgaris* petioles however did not exhibit cell division at the separation layer before cell separation. Bornmann *et al.* (1968) also reported cell division in the stem abscission zones of *Gossypium hirsutum* L..

The aim of the present investigation was to compare anatomical and ultrastructural changes occurring during induced adventitious abscission of *Impatiens sultani*, *Ipomoea batatas* and *Begonia corallina* internodal explants, and also to compare these processes with such changes reported to occur in normal organ abscission such as leaf abscission.

The terminology used is that recommended by Addicott (1982, pp 20-21). Thus, the separation layer is a narrow layer of separating cells, both in normal and adventitious abscission. Abscission zone which includes the separation layer is a

more substantial region found at the base of plant organs (such as leaves, fruits, flowers) and is the site of normal abscission. A protective layer forms by the modification of the cells (e.g. suberization) on the scar surface after separation. The protective layer development may involve cell division either before or after separation.

2. MATERIALS AND METHODS

Impatiens sultani Hook., *Ipomoea batatas* (L.) Lam. and *Begonia corallina* Carriere plants were grown and the explants obtained from them were cultured in the following media as described in Chapter 1.

(1) *Impatiens sultani*: 0.0001% IAA ± 0.0001% zeatin + 1.0% sucrose

(2) *Ipomoea batatas*: 0.0001% IAA ± 0.0001% zeatin + 3.0% sucrose

(3) *Begonia corallina*: 0.01% IAA ± 0.0001% zeatin + 3.0% sucrose

In addition, *Impatiens sultani* and *Ipomoea batatas* were cultured in 1% sucrose and 3% sucrose media respectively (without added hormones), and ethylene at a concentration of 10 µl/l was included into the explant environment 1 day after explanting (for details see Chapter 1). All explants were incubated at 26^{o}C in the dark.

At various stages of abscission appropriate regions of the internodal segments were sliced longitudinally into 0.5 mm thick pieces under 3.0% glutaraldehyde in 25 mM cacodylate buffer, at pH 7.0. The tissue slices were fixed in the same fixative on ice for 2 h, post-fixed in 1% osmium tetroxide in 50 mM cacodylate buffer for 3 h at

room temperature, dehydrated in either ethanol or acetone and were embedded in either L.R. White (London Resin Company) or Epon-Araldite.

For electron microscopy, 75-100 nm thick sections were taken on a Reichert Ultratome ultramicrotome, stained with uranyl acetate followed by lead citrate (Reynolds 1963), and observed using Hitachi H600 or JEOL 2000EX electron microscope at 80kV or 120 kV respectively.

For light microscopy, 1-2 μm thick, resin embedded sections were stained with toluidine blue O or $KMnO_4$ (O'Brien and McCully 1981).

For scanning electron microscopy, abscised explant segments were quick frozen in liquid nitrogen and were observed with or without gold coating, under a Cambridge Stereoscan 380 microscope, equipped with a Hexland cold stage.

3. RESULTS

3.1. *Impatiens sultani*

Figure 1 illustrates the progressive stages of abscission, from explanting to separation of the upper segment. The position of future separation layer became externally visible as a faint silvery line encircling the explant, several millimeters above the base, 3-4 days after explanting. Within 12-24 h of the appearance of this line the upper segment separated from the lower segment completing abscission.

3.1.1. Light microscopy

The first noticeable anatomical modification also occurred around 3 days after explanting, in the inner cortex close to the vascular bundles (internodes used do not show interfascicular development and vascular bundles are separated by broad parenchymatous rays). In these cells the walls appeared somewhat distorted and the cytoplasm often appeared plasmolysed (Fig. 8). Eventually the cells separated, and the process spread outwards through the collenchymatous outer cortex and the epidermis and lastly into the pith (Fig. 9). The separation layer formed is morphologically upwardly concave (i.e. it spreads downwards in the pith) in longitudinal section. Some xylem vessels close to the separation layer became blocked by tyloses prior to abscission and most tyloses were on the proximal side of the separation layer (Fig. 10).

In ethylene induced abscission, often the separation layer area appeared swollen externally. Anatomical observations of light microscopic sections of the outer cortical cells at the separation layer showed enlargement, similar to the fanning out of cells at the separation layer, reported by Sexton and Redshaw (1981) for *Impatiens sultani* leaf abscission zones.

The proximal fracture surface, 2 days after abscission demonstrated a layer of cells, positively staining with $KMnO_4$ immediately below the separation layer cells (Fig. 11).

3.1.2. Transmission electron microscopy

Ultrastructurally, the cells of the abscission site showed increased activity of the endomembrane system (Figs 13, 15) at the outset of abscission (3 days after explanting), when compared to cells away from the abscission site (Fig. 12). Both rough endoplasmic reticulum (RER) and the number of dictyosomes in the cells were found to increase (Fig. 13). Dictyosomal vesicles originating from the secreting phase (trans-phase) were seen fusing with the plasmalemma (Fig. 14).

With the progress of abscission, the cytoplasm of the abscission zone cells became less electron-dense, and the ribosomes became less prominent (Figs 16, 17). Dictyosomes appeared more dilated and were found to be associated with a large number of vesicles at different stages of development (Fig. 17). The forming phase and the secretory phase of the dictyosomes were indistinguishable at this stage and the plasmalemma appeared convoluted (Fig. 17). These ultrastructural changes are common to most parenchymatous cells of the pith and cortex, as well as collenchyma of the outer cortex (Fig. 17), on both distal and proximal sides of the fracture surface.

Cell separation became evident ultrastructurally with the dissolution of the middle lamella (Fig. 16) and microfibril separation (Fig. 18). The cell separation is confined to 3-4 layers of cells and the separated cells were lined with loose cell wall microfibrillar material on the outside and a dispersion of microfibrils were found between the cells (Figs 20, 21). The plasmalemma and the tonoplast membranes were found to be intact even in these separated cells (Fig. 21). The separated cells in the pith often appeared spherical (Fig. 22).

3.1.3. Scanning electron microscopy

Almost all the cells on the proximal and distal sides of the fracture surface except in the vascular area, appeared to have separated from each other along the middle lamella (Figs 23, 28, 29). Further examination of the fracture surfaces revealed that most of these cells were intact and turgid (Figs 24, 25) as observed previously by Warren Wilson *et al.*(1986). Most cells in the pith appeared rounded and were attached to neighbouring cells only by a small area of contact (Figs 24, 25). Often gelatinous cell wall degradatory products were seen on the separated cells, and also in the spaces between these cells (Fig. 25). Occasionally calcium oxalate crystals were observed dispersed among cell wall degradatory products (Fig. 26). The xylem vessels in the vascular region appeared ruptured, presumably due to mechanical force during final separation of the upper segment. The pulled out spiral thickenings of these vessels were often seen in the vascular areas (Figs 29, 30). The parenchymatous cells of the vascular region however separate without rupturing, like cells of the other regions of the separation layer (Fig. 30). The distal fracture surface showed depressions representing vascular regions (Fig. 23). Since the separation layer tends to spread upwards in the cortex, it produced a narrow frill (Lloyd 1914; Warren Wilson *et al.* 1986), composed of epidermis and outer cortical collenchymatous cells, along the edge of the lower segment (Fig. 31). Most epidermal and thick walled collenchymatous cells were also found to be intact after abscission (Figs 27, 31).

3.2. *Begonia corallina*

Before the site of abscission became externally visible, the upper region of the explant showed signs of senescence. Typically it started to turn brown beginning from the upper end at the onset of separation layer development (Fig. 3). At the time of abscission a greater part of the distal segment had turned brown (Fig. 4). However, when the separation layer formed rapidly (4-5 days after explanting), only a very slight colour change was evident in the upper region. Then the epidermis of the upper segment took a silvery gray tinge before abscission.

3.2.1. Light microscopy

Cell separation along the middle lamella was very prominent at the separation layer (Figs 32, 34). Occasionally in the pith, cell separation spreads upward to 8-10 cell layers and formed a 'fluffy' mass of loosely attached cells above the fracture plane. Rarely the separation layer also had the tendency to move upward along the vascular region (Fig. 34) instead of passing through the latter into the cortex. The cells beneath the epidermis in the distal segment appeared distorted and degraded at the time of abscission (Fig. 33).

3.2.2. Transmission electron microscopy

Ultrastructurally, separation layer cells in both pith and cortex were similar to those of *Impatiens sultani* in exhibiting increased activity of dictyosomes (Figs 35, 36). At later stages of abscission the plasmalemma appeared highly convoluted and the cytoplasm contained increased number of vesicles (Figs 37, 39, 40). Some cells

also showed increased number of microtubules (Fig. 37). When cell separation spread to more cell layers in the pith, separation always initiate at the intercellular spaces (Fig. 38). These cells did not show the active endomembrane system characteristic of separation layer cells, and presumably cell separation in these cells is achieved by the diffusion of hydrolyzing enzymes from the separation layer, through intercellular spaces and cell walls. The intercellular spaces sometimes contained vesicle like material (Fig. 38).

3.3. *Ipomoea batatas*

Figure 2 shows the progressive stages of abscission from explanting. After 4-5 days of explanting the region above the separation layer started to turn yellow and at abscission a greater part of the upper segment had turned yellow.

Some *Ipomoea batatas* internodal explants (especially very young internodes) occasionally developed an unusual upper separation layer (Fig. 5), 0.5-5.0 mm below the distal end (2-3 days after explanting), before the development of the lower separation layer which forms 4-5 mm above the base of the explant. This upper separation layer occasionally has the tendancy to develop diagonally (instead of transversely) across the stem (Figs 6,7).

3.3.1. Light microscopy

The first noticeable anatomical change in the abscission region was the appearance of a layer of newly divided cells, close to the vascular region. Later cell division spread down into the pith and the cortex (Figs 41, 42). The process of cell

division occurs in such a way that it produced a layer of compressed cells (with very few intercellular spaces) across the internode, which was saddle shaped in longitudinal section (Fig. 43). As in *Impatiens* the separation layer is displaced upwards in the vascular region (Figs 43, 45). Cell division was also prominent in the parenchymatous cells of the vascular area (Fig. 46). Tylose formation in xylem vessels occurred during early stages of separation layer development. These are fairly restricted to the separation layer area (mostly within 800 µm of separation layer; Fig. 44). The anatomy of the upper separation layer was closely similar to the lower separation layer. However, occasionally it tend to develop diagonally instead of transversely across the internode (Fig. 45).

The final cell separation was confined to the distal region of the zone formed by cell division, and the fracture usually occurred between the non-divided cells and the newly divided cells, thus confining the latter to the lower segment (Figs 45, 46).

In ethylene induced abscission, the separation layer cells in the cortex showed enlargement (Figs 47, 48), close to abscission.

3.3.2. Transmission Electron microscopy

Ultrastructurally, both newly divided cells and the non-divided cells of the separation layer showed increased endomembrane activity when compared to cells away from the separation layer (Fig. 49). Figures 50 and 51 illustrate cells at the onset of cell division (4 days after explanting), at the abscission site. Figures 52-56 are at a later stage (5 days after explanting) which show middle lamella dissolution

and microfibrillar separation. Subsequent cell separation (which is confined to only 2-3 layers of cells) is also clearly evident.

The cells illustrated in Figs 52-53 were from the proximal side of the fracture plane. Although the newly formed thin cell walls appeared relatively unaffected, the older thick cell walls appeared to be separating with a dispersion of loose cell wall microfibrillar material filling the space in between the cells. Figures 54-56 illustrate a separated cell on the distal side of the fracture plane, which also showed increased dictyosomal activity and a highly convoluted plasmalemma. It also showed a high density of microtubules (Fig. 56). As in *Impatiens sultani* RER was prominent during early stages of abscission (Figs 50, 51) and this later became dilated (Figs 53, 55). Tylose blocked xylem vessels could also be observed close to the separation layer (Fig. 57). Ultrastructurally the cells of the upper separation layer appeared identical to those of the lower separation layer.

3.3.3. Scanning electron microscopy

In *Ipomoea batatas* often the upper segment did not completely separate and fall off from the lower segment (the pith and the vascular region failed to separate completely). But when complete separation occurred, examination of the fracture surface revealed that most of the cells were intact (Figs 58-62). However, some pith cells were found to be ruptured (Fig. 59). This is probably due to mechanical damage to the thin walls of newly divided cells, during the separation of the upper segment. Material of a gelatinous nature (Fig. 62), and globular deposits (Fig. 61), most probably representing cell wall degradatory material were also observed on

separated cells. In addition some dense granular lumps of material were also observed on these cells, prominently on the outer cortical cells (Fig. 62). This may represent more cell wall degradatory products or latex.

3.4. Some general observations

The form of the separation layer and the ultrastructural details of its cells were similar in all three species irrespective of the type of medium used to induce abscission (0.0001% IAA or 0.0001% IAA + 0.0001% zeatin) including that of abscission induced by ethylene (except in *Begonia* which was not examined). However, in ethylene induced abscission of *Impatiens sultani* and *Ipomoea batatas*, the cortical cells demonstrated enlargement (especially in *Ipomoea batatas*) prior to abscission.

4. DISCUSSION

The results show that in all three species abscission is achieved primarily by the dissolution of the middle lamella, and to some extent the cell wall of the separation layer cells, leading to cell separation. Such complete cell separation was observed in all different cell types across the internode except in xylem vessels. Xylem vessels on the other hand were often found ruptured during abscission. In all three species no cell breakdown (except xylem vessels) was observed, this contrasts with the observations made for adventitious abscission of *Phaseolus vulgaris* stem explants (Webster and Leopold 1972). However, in *Ipomoea batatas* the cell separation

sometimes did not complete, especially in the pith and vascular region. This was somewhat comparable to the incomplete adventitious abscission reported for *Phaseolus* pedicels (Osborne and McManus 1986) and apple pedicels (Pierik 1977).

The process of cell separation was typically confined to 2-4 cell layers as in leaf abscission zones (Sexton and Roberts 1982). However, in *Begonia corallina* the cell separation process sometimes spreads to 8-10 cell layers in the pith during the later stages of abscission.

Abscission in *Ipomoea batatas* was distinctly different from that in the other two species studied, in showing cell division in the abscission site, prior to separation, with the newly divided cells being confined to the lower segment after abscission. A comparable process of cell division was reported to be characteristic of normal abscission (e.g.leaf) in many species (Gawardi and Avery 1950; Halliday and Wangermann 1972; Sexton and Hall 1974; Sexton and Roberts 1982) and also in adventitious abscission (Webster and Leopold 1972). Gawardi and Avery (1950) suggested that in leaf abscission, cell division is not essential for the actual cell separation (abscission) process. Similarly in *Ipomoea batatas* explants the primary function of these newly divided cells may presumably be to form a protective layer to the exposed fracture surface. The fact that internodal abscission in *Impatiens sultani* and *Begonia corallina* occurred without cell division at the abscission zone also suggest that the cell division when it occurs may not be contributory to the process of cell separation. In *Phaseolus vulgaris* internodal abscission, cell division was preceded by prominent cell breakdown (Webster and Leopold 1972). In *Ipomoea*

batatas by contrast, cell division was the first noticeable anatomical modification which may be indicative of an early protective layer formation.

The form of these internodal separation layers is interesting, and may reflect the physiology of positional definition of adventitious abscission layer formation (see Warren Wilson *et al.* 1986). In *Impatiens sultani* and *Ipomoea batatas* the separation layer was found to be concave in shape (i.e. it is displaced downwards), in the pith (in *Begonia corallina* this is not so evident). It is suggested that IAA acts as a morphogen, in defining the position of the separation layer, above the basal accumulation of auxin, due to polar auxin transport (Warren Wilson *et al.* 1986). On this assumption, it could be proposed that the concave shape of the abscission zone is a result of smaller quantities of auxin in the pith region.

Ultrastructurally, these adventitious abscission zone cells were very similar to normal leaf abscission zone cells, which had been reported earlier (Osborne and Sargent 1976; Sexton 1976; Sexton *et al.* 1977; Sexton and Roberts 1982). All three species showed marked increases in the activity of dictyosomes in the separation layer cells. Rough endoplasmic reticulum was also prominent especially during early stages. This increased cytoplasmic activity was observed in most of the cell types found in the separation layer which included pith parenchyma, cortical parenchyma and collenchyma, and parenchymatous cells of the vascular region. As suggested for leaf abscission zone cells (Sexton and Roberts 1982), this active endomembrane system is presumably involved in the production and transport of cell wall degrading enzymes across the plasmalemma. It is interesting that in all three species the plasmalemma appeared highly convoluted when the dictyosomes were very active,

especially in *Begonia corallina* (Fig. 40), which exhibited extensive cell separation. This was indicative of continuous fusion of dictyosomal vesicles with the plasmalemma.

The increased dictyosomal activity observed in newly divided cells in the separation layer of *Ipomoea batatas* could be relics of wall synthesis during cell division, as proposed by Sexton *et al.* (1977) for similar observations made on *Coleus* leaf abscission. On the other hand, these active dictyosomes may also secrete cell wall hydrolyzing enzymes needed for cell separation, especially during later stages, as in abscission zone cells of *Impatiens sultani* and *Begonia corallina*, where cell division does not occur. The seemingly very active dictyosomes in non-divided cells in the distal region of the separation layer of *Ipomoea batatas* could also be considered as involved in such hydrolytic enzyme secretion.

Microtubule numbers increased somewhat in the separation layer cells of all three species. Although microtubules can guide the movement of vesicles in animal cells (Hyams and Stebbings 1979; Hayden and Allen 1984), Sexton and Redshaw (1981) reported that the microtubule depolymerizer colchicine had no effect on cell separation, and concluded that microtubules were not essential for *Impatiens sultani* leaf abscission. Since microtubule numbers observed in *Impatiens sultani* internodal separation layer cells were much smaller than those observed in leaf abscission zone cells by Sexton and Redshaw (1981), their function may not be crucial for the adventitious abscission process.

Ipomoea batatas and *Impatiens sultani* xylem vessels frequently show tylose formation close to the separation layer (Figs 10, 44). Association of tyloses with leaf

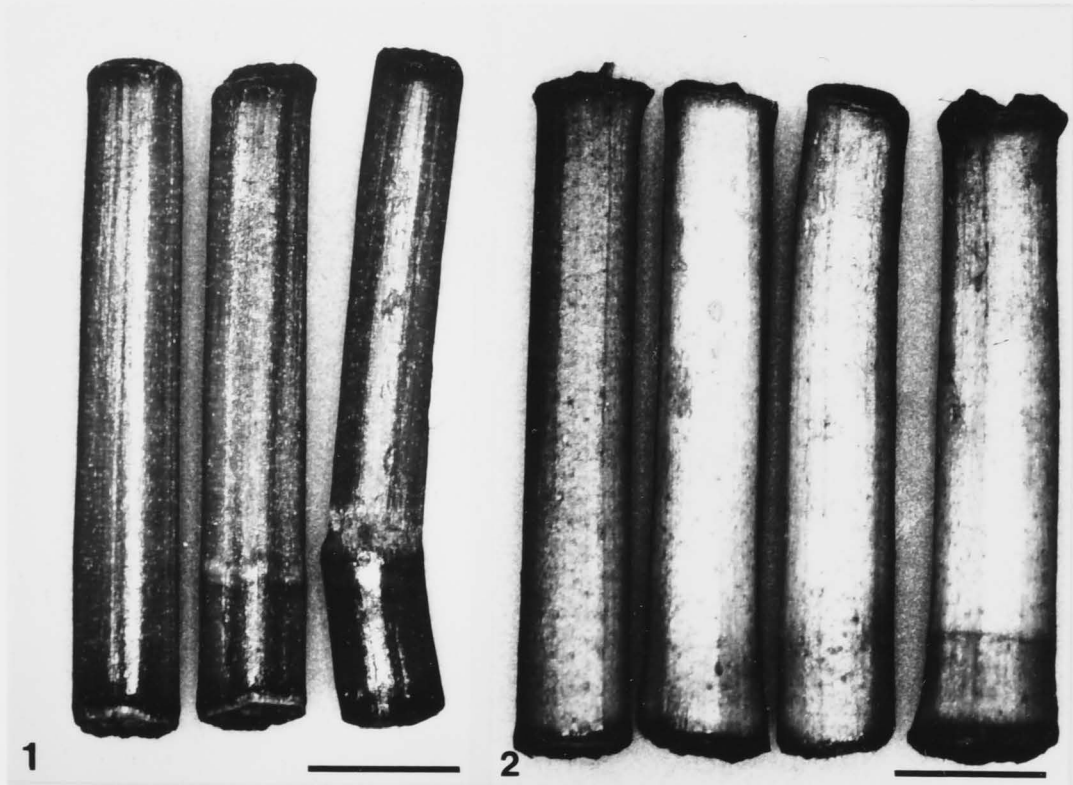
abscission zones have been widely reported in the literature, however, their role in the abscission process remains unclear. Their proposed roles include; weakening the xylem vessel walls, causing water stress and shrinkage of the distal tissues, and inhibition of pathogen entry (Sexton and Roberts 1982). Bornmann (1967) had suggested that tyloses do not play a role in abscission, since the number of tyloses formed was inversely related to speed of abscission. In the internodal abscission studied here, tylose formation was not common to all vessel elements (especially in *Impatiens*), and there were no visible signs of water stress or shrinkage of the distal segment of the internode. Hence, tylose formation may not play a major role in the process of internodal abscission in *Impatiens*. However, in *Ipomoea* tylose formation was more prominent and localized to the abscission site and may aid in the weakening of the xylem vessel walls at the separation layer. The fast yellowing of the upper segment observed in *Ipomoea batatas*, as compared to the other two species studied, could also be attributed to tylose formation, in addition to possible interruption of metabolite transport due to early protective layer formation across the internode.

Ipomoea batatas (and *Impatiens sultani* to a lesser extent) demonstrated enlargement of separation layer cells before abscission, when abscission was induced by ethylene but not by IAA or IAA + zeatin. This was comparable to ethylene induced expansion growth of cells in *Phaseolus vulgaris* leaf abscission zone reported by Wright and Osborne (1974). Such cell expansion at the separation layer has been suggested to aid in the separation of the abscising organ or the explant segment (Wright and Osborne 1974; Sexton and Redshaw 1981).

Fig. 1. *Impatiens sultani*: progressive stages of abscission (initial, 4 days and 5 days after explanting). Scale bar = 5mm

Fig. 2. *Ipomoea batatas*: progressive stages of abscission (initial, 3 days, 4 days, and 6 days after explanting).
Scale bar = 5mm

Figs 3-4. *Begonia corallina*: two stages during abscission. Fig. 3: 5 days after explanting, Fig. 4: 7 days after explanting.
Scale bar = 5mm.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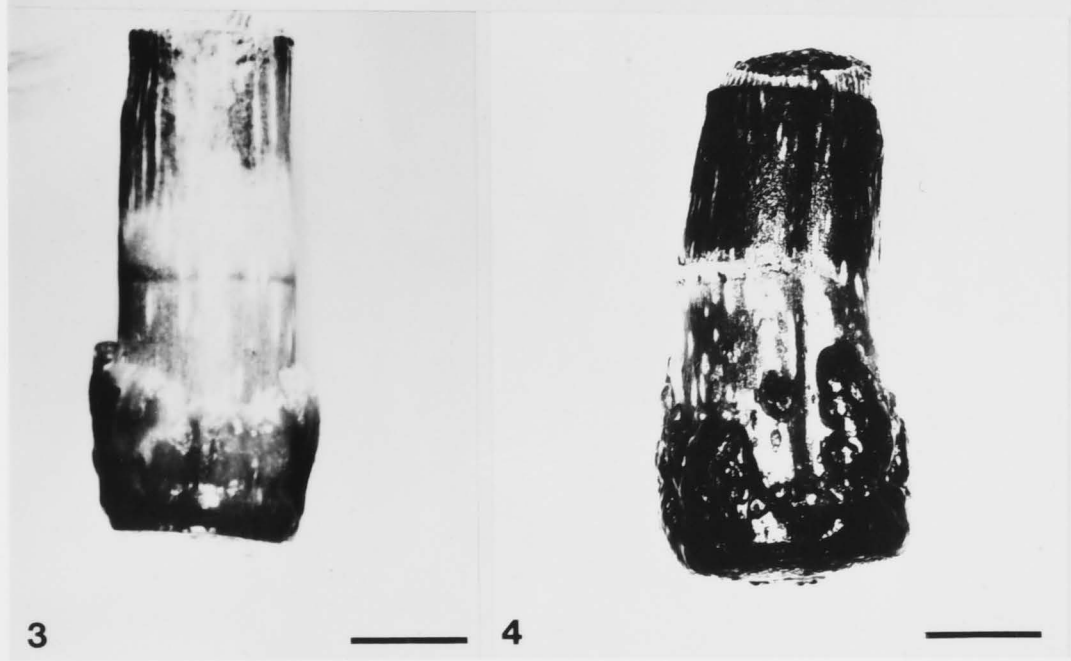
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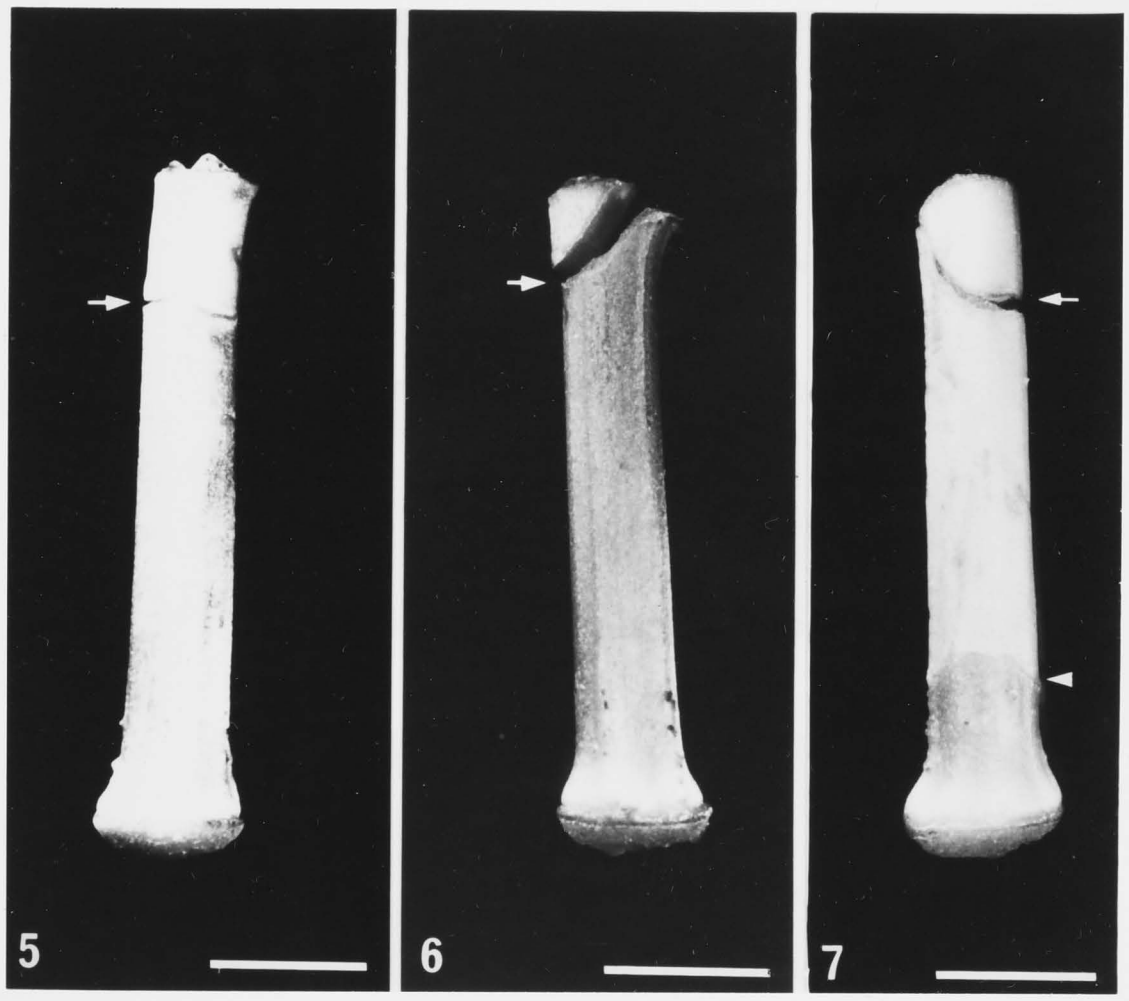
Figs 5-7. *Ipomoea batatas*: Different morphological forms of the upper separation layer (arrow), lower separation layer (arrowhead). Scale bar = 5mm.

Fig. 5. A transverse upper separation layer formed 3 days after explanting.

Fig. 6. An upper separation layer that had developed diagonally (4 days after explanting).

Fig. 7. This explant shows both a diagonally developed upper separation layer and the transverse lower separation layer that had devloped later (6 days after explanting).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Figures 8-11. *Impatiens sultani*: Light micrographs of longitudinal sections through the separation layer area. (Col = collenchyma; Co = cortex; Pi = pith; small arrow heads = separating cells).

Fig. 8. Distorted cells of the inner cortex (large arrowheads) 3 days after explanting.

Scale bar = 100μm.

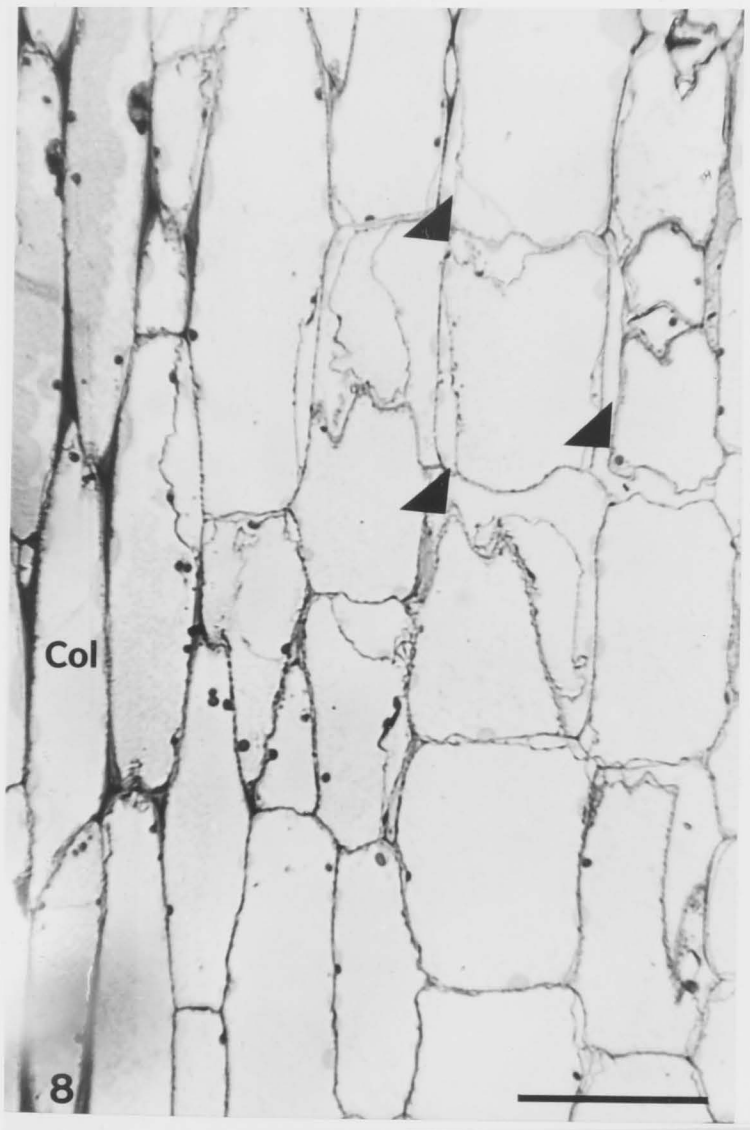
Fig. 9. The development of the separation layer in the cortex at a later stage (4 days after explanting). The double headed arrow indicates the direction of fracture. Scale bar = 200μm.

Fig. 10. Association of tylose (Ty) filled xylem vessels close to the separation layer.

Scale bar = 100μm.

Fig. 11. $KMnO_4$ stained cells on the proximal fracture surface, 2 days after abscission. Note the heavy staining (arrows) of the cells immediately below the cells at the separation layer (SL).

Scale bar = 100 μm.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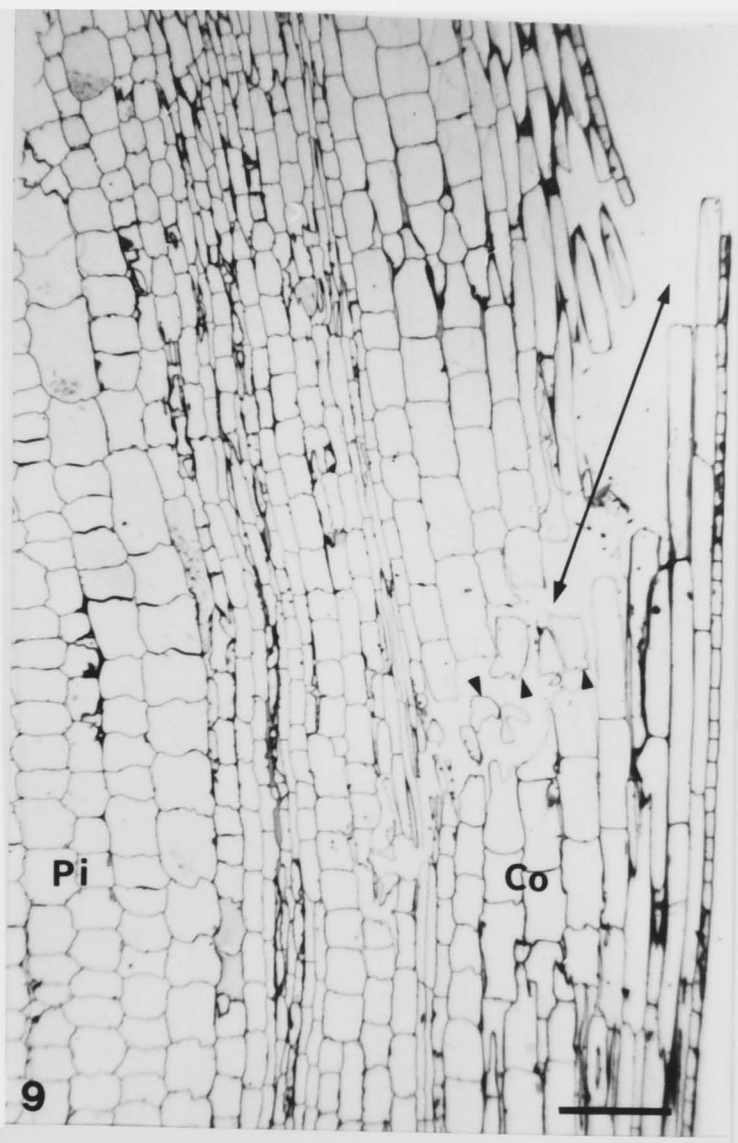
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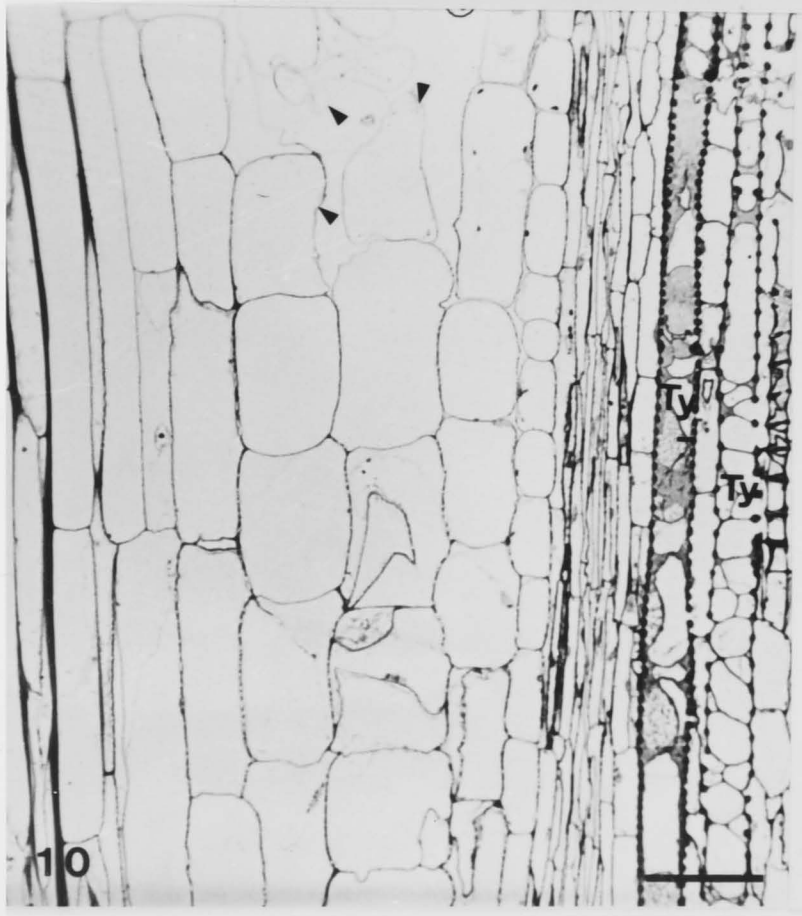
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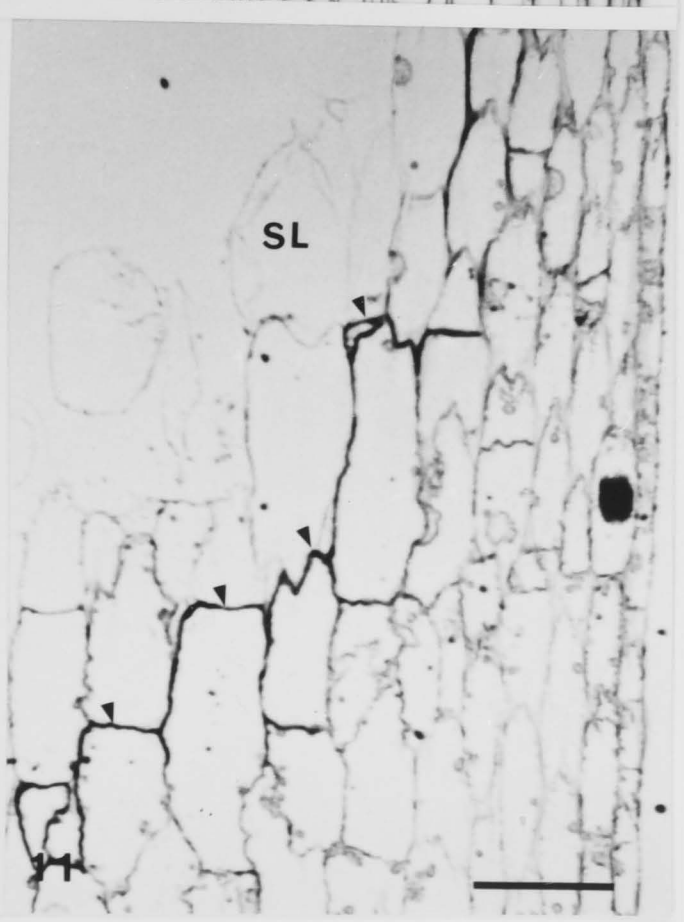
SL
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Fig. 12. *Impatiens sultani*: electron micrograph of a cortical cell 5-6 cells below the separation layer (4 days after explanting). Note the absence of active dictyosomes.

Scale bar = 2μm.

Figures 13-22. *Impatiens sultani*: Ultrastructure of separation layer cells. (D = dictyosomes; RER = rough endoplasmic reticulum; V = dictyosomal vesicles).

Figures 13-15. Inner-cortical cell cytoplasm at the onset of separation layer formation (3 days after explanting).

Fig. 13. Note the increased endomembrane activity.

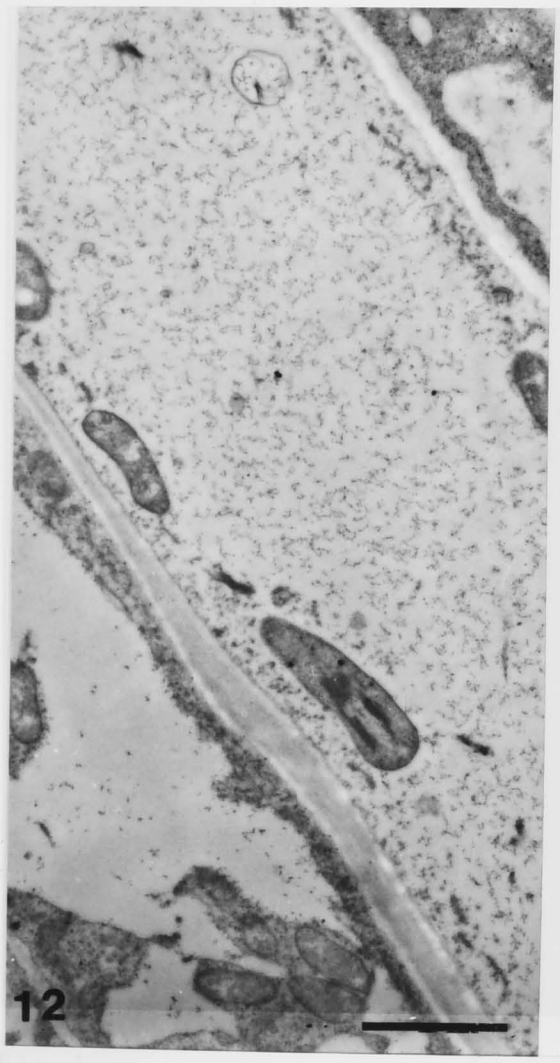
Scale bar = 1μm.

Fig. 14. Note the dictyosomal vesicles (V) fusing with the plasmalemma, and prominent, ribosome bearing rough endoplasmic reticulum (RER).

Scale bar = 0.5μm.

Fig. 15. Note the abundance of rough endoplasmic reticulum (RER).

Scale bar = 1μm.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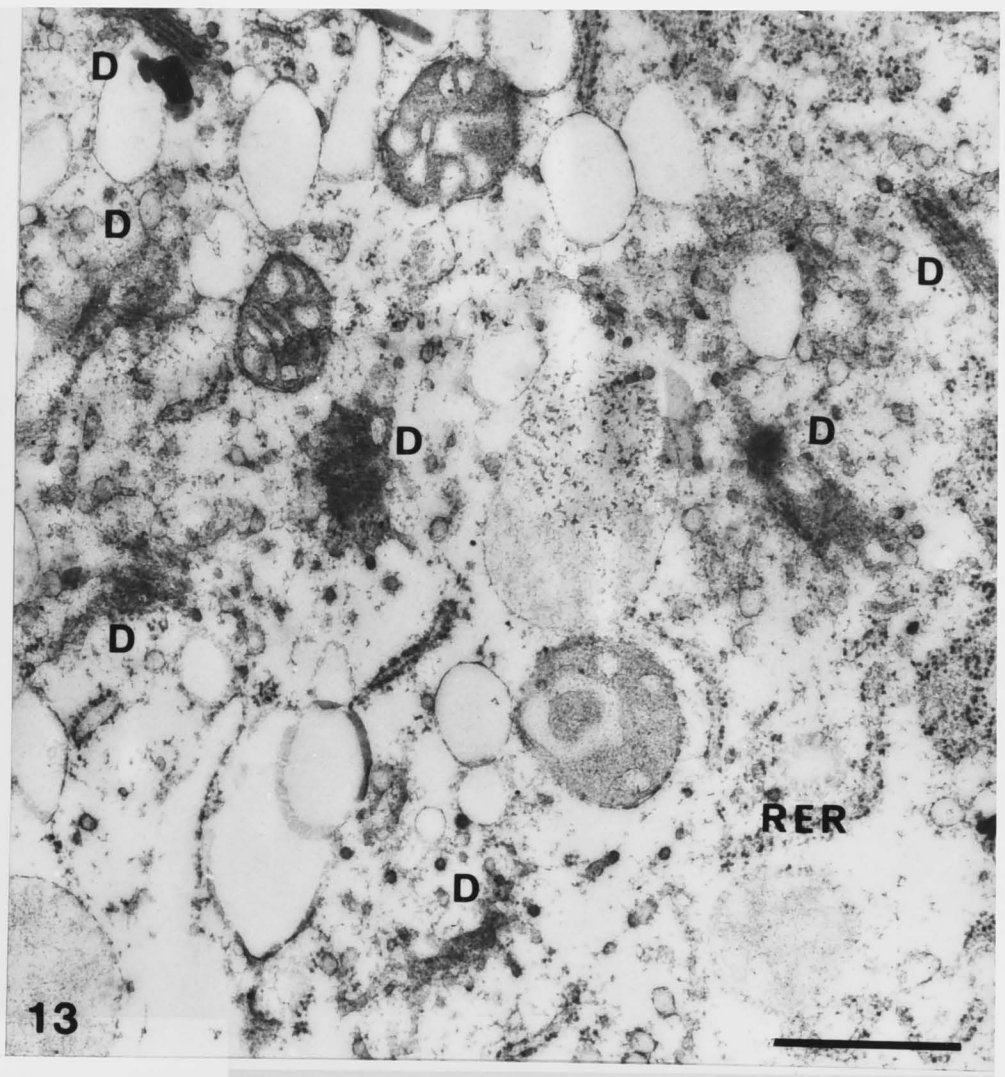
D
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RER
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RER
V
D
14


RER
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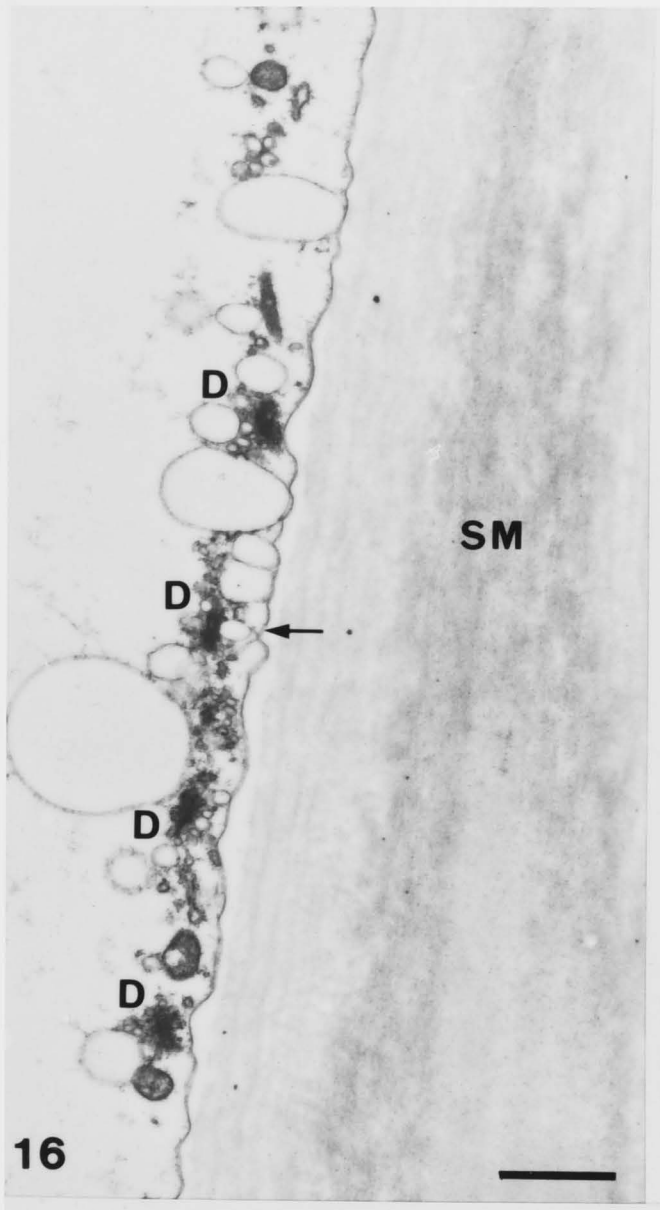
Figs 16-17. An outer cortical collenchyma cell at the separation layer (4 days after explanting). Note the separating microfibrils (SM) in the middle lamella region and active dictyosomes associated with numerous vesicles (V). The plasmalemma appear convoluted (arrow). Fig. 17 (Scale bar = 0.5μm) is an enlarged area of Fig. 16 (Scale bar = 1.0μm).

Fig. 18. Separating cellulose microfibrils in the pith cells (arrowhead), 4 days after explanting.

Scale bar = 0.5μm.

Fig. 19. Tylose (Ty) filled xylem vessel close to the separation layer, 4 days after explanting.



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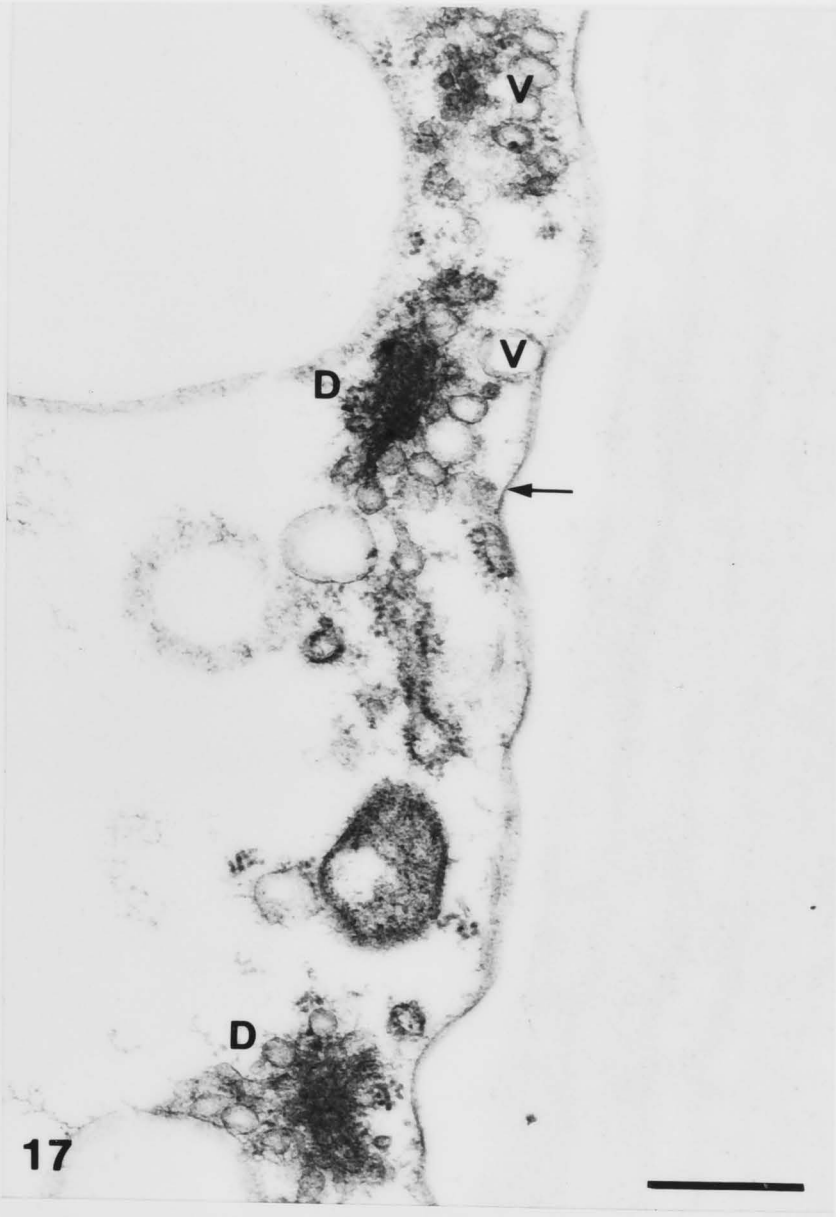

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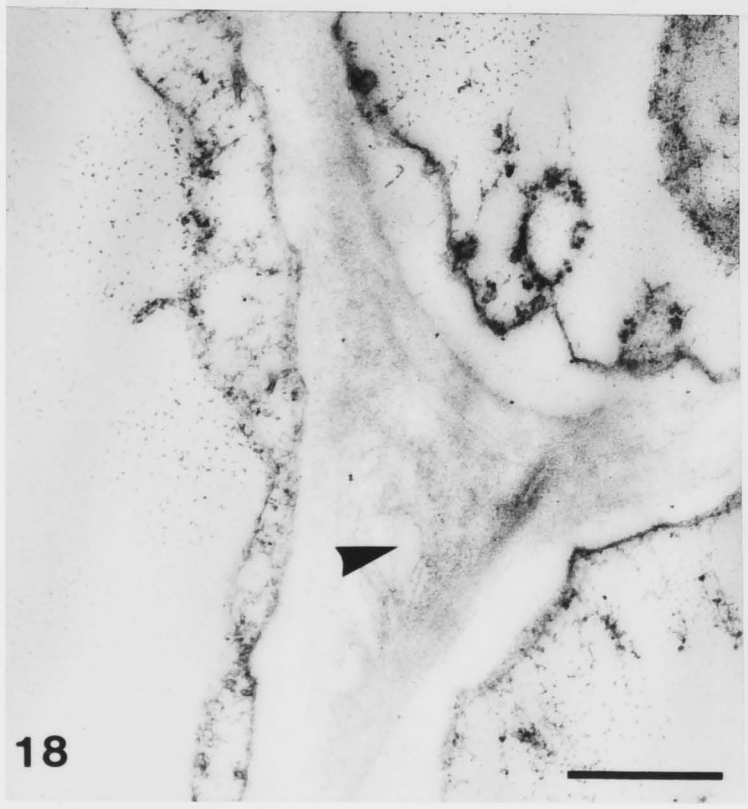

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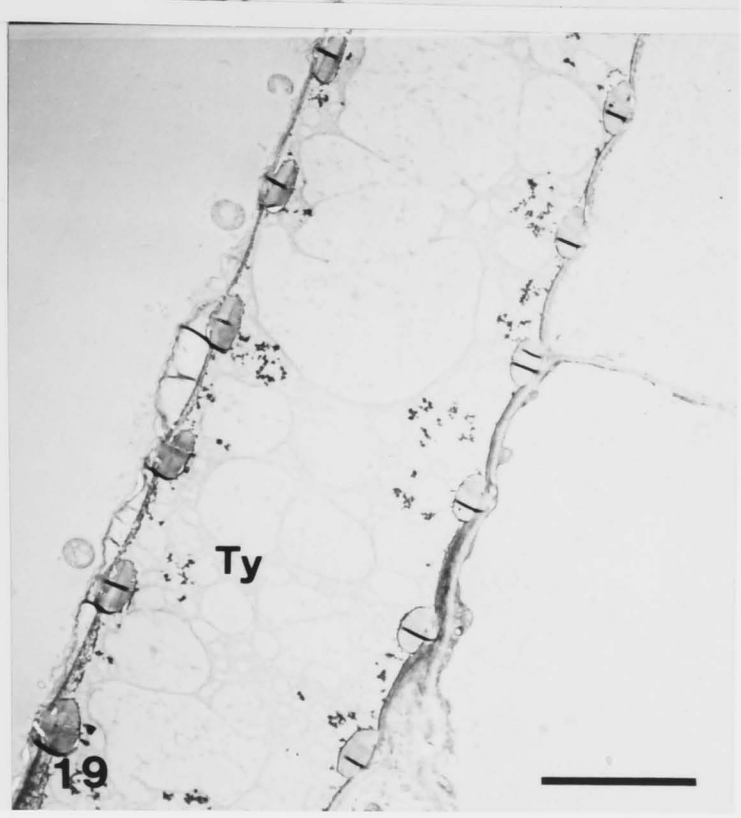

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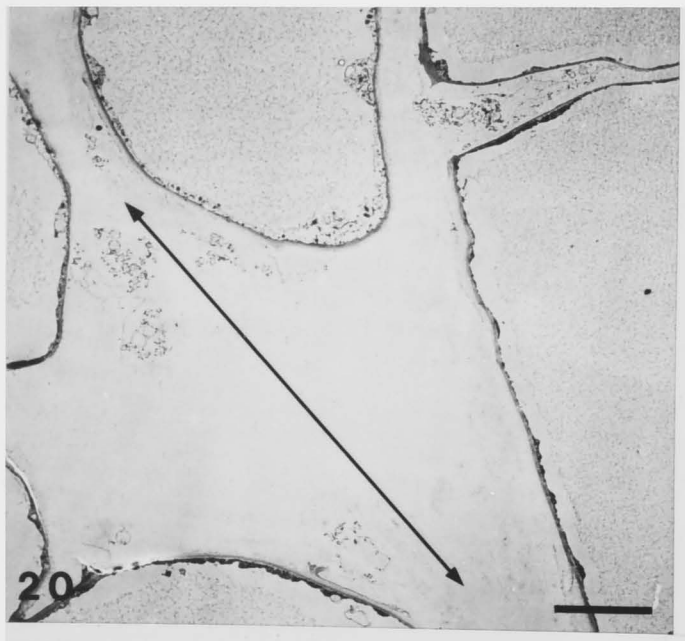
Figs 20-21. Separating inner cortical cells, 4 days after explanting. Double sided arrow indicate the direction of fracture. Note the dispersed cell wall microfibrils (DM) lying between separated cells, and the intact plasmalemma and the tonoplast membranes (Fig. 21). Fig. 21 is an enlarged area of Fig. 20.

Scale bar = 10μm.

Fig. 22. A separated pith cell exhibiting a rounded shape (5 days after explanting). Scale bar = 10μm.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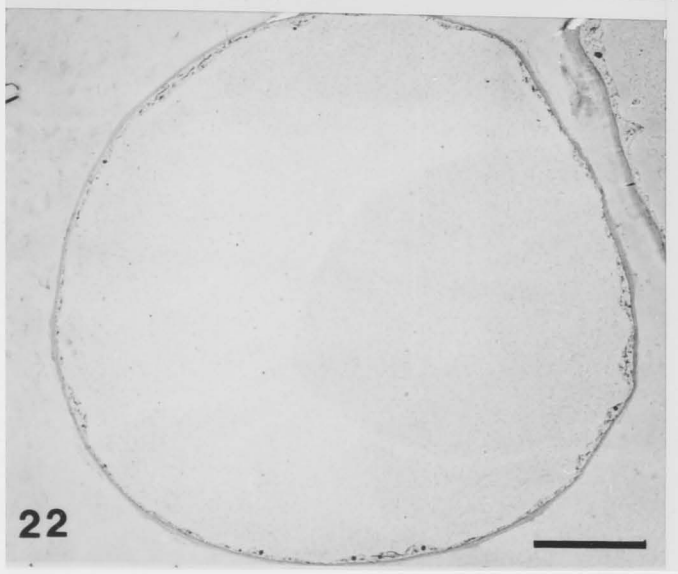
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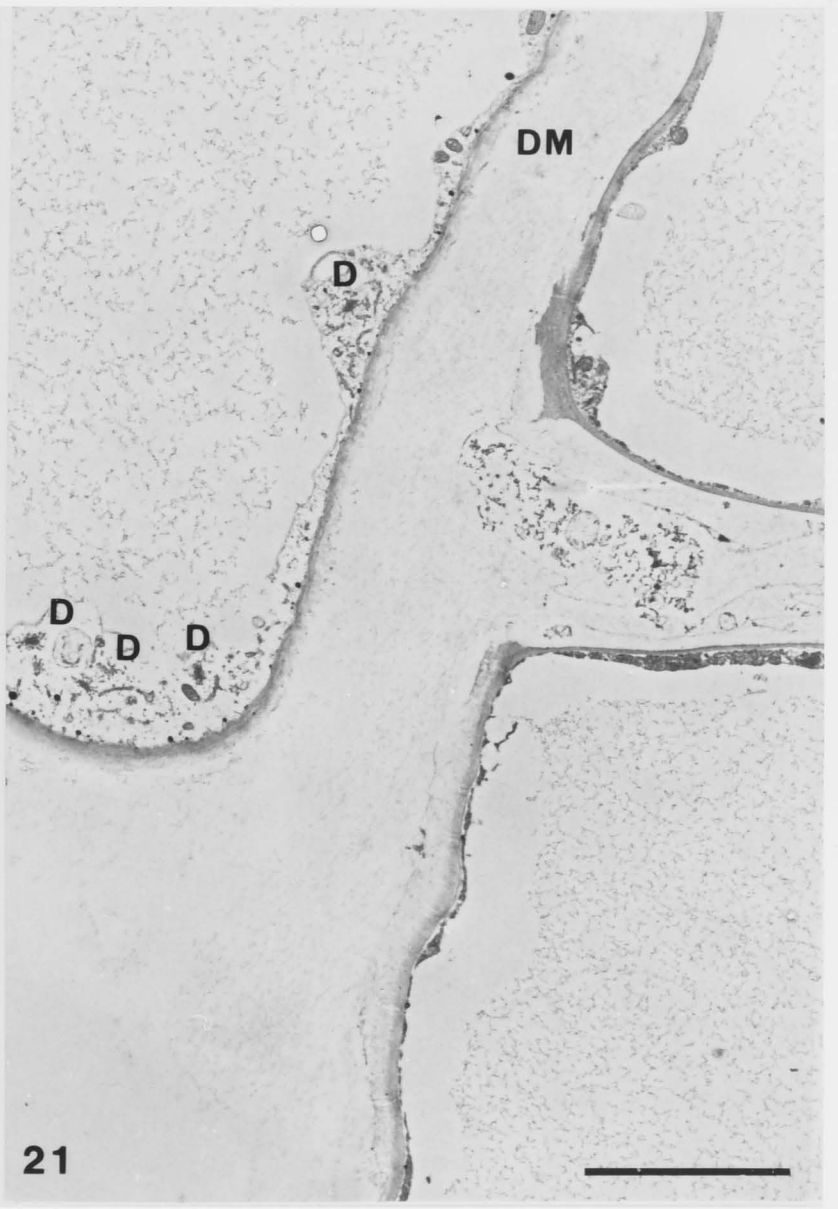
DM
D
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Figures 23-27. Scanning electron micrographs of *Impatiens sultani*, distal fracture surface after abscission.

Fig. 23. Note the intact and rounded nature of most cells and the depression of fracture plane in the vascular area (arrowhead). Scale bar = 250μm.

Figs 24-25. Micrographs of pith cells in Fig. 23 at a higher magnification. Note the intact and turgid nature of the cells and deposits of gelatinous nature on and between cells (arrows).

Fig. 24. Scale bar = 100μm.

Fig. 25. Scale bar = 50μm.

Fig. 26. Cortical cells with dispersed calcium oxalate crystals. Scale bar = 50μm.

Fig. 27. Intact epidermal and collenchymatous cells (Col) at the fracture surface (Ep = epidermis).

Scale bar = 50μm.


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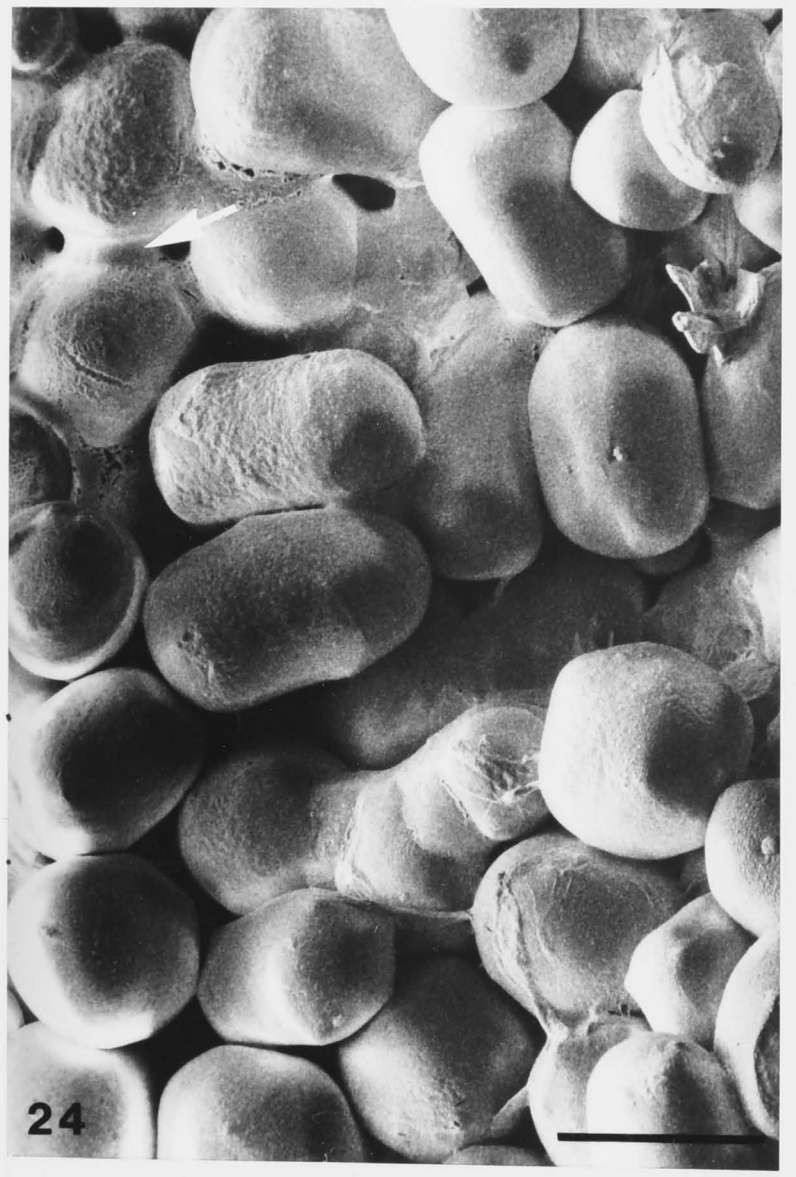
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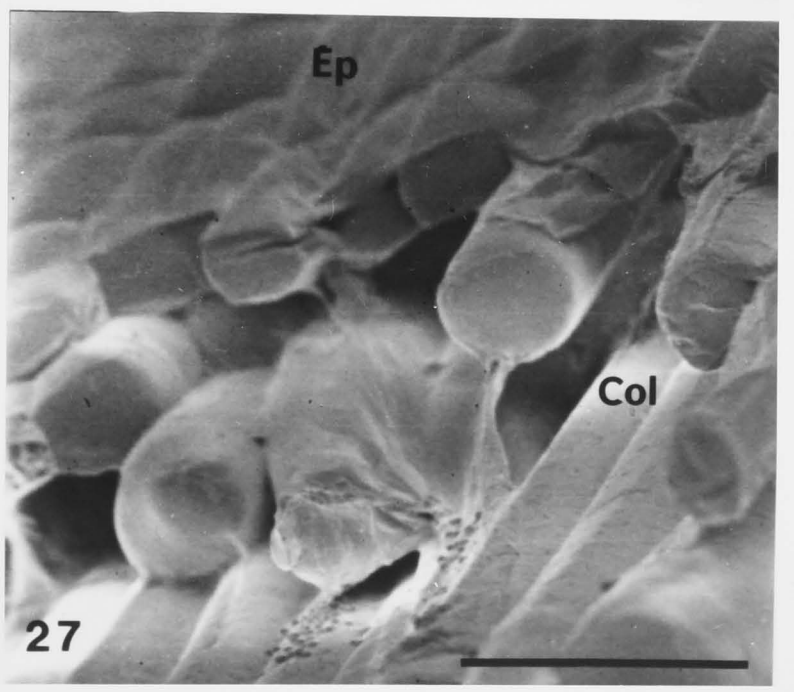
Ep
Col
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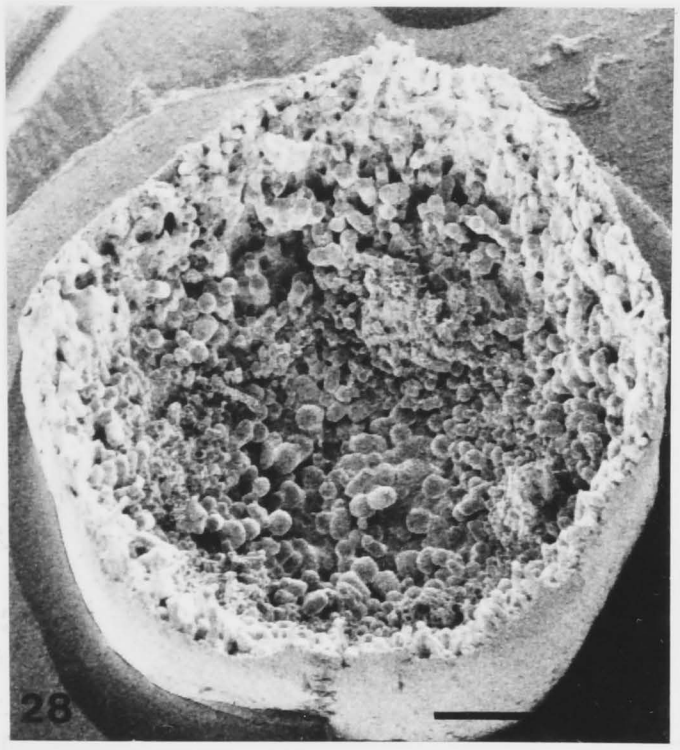
Figures 28-30. Scanning electron micrographs of *Impatiens sultani* proximal fracture surface after abscission. Note the intact nature of the cells and drawn out spiral thickening of the xylem vessels (arrows = spiral thickenings; arrowheads = fractured xylem vessel). Figs 29-30 are enlarged areas from Fig. 28.

Figs 28-29. Scale bar = 400μm.

Fig. 30. Scale bar = 100μm.

Fig. 31. A side view of the proximal segment of *Impatiens sultani* after abscission. Note the frill formed by the epidermis and collenchymatous cells.

Scale bar = 400μm.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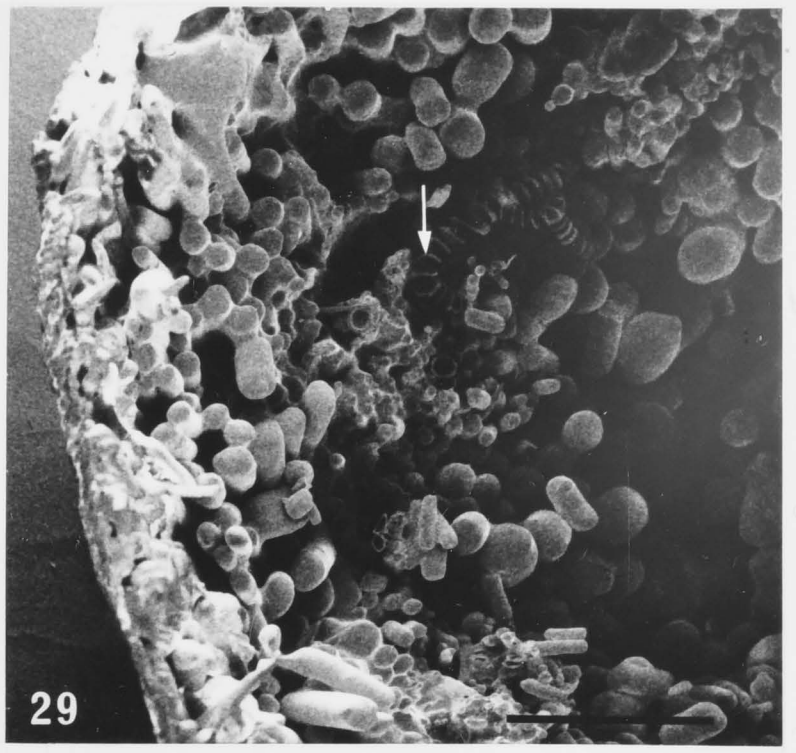
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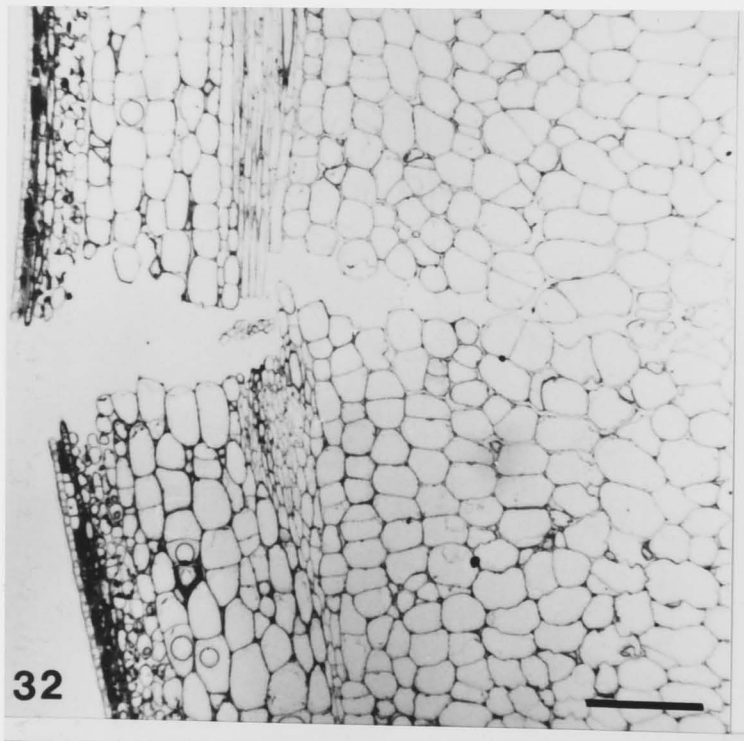
30


31

Fig. 32-33. Course of *Begonia corallina* separation layer close to completion of abscission (7 days after explanting). Note the intact and separated cells at the separation layer and distorted and disintegrating cells beneath the epidermis in the upper segment (arrow). Fig. 33 (Scale bar = 200μm) is an enlargement of Fig. 32 (Scale bar = 400μm)

Fig. 34. The course of the separation layer of *Begonia corallina* when on rare occasions it spreads upwards along the vascular area. Double sided arrow denotes the direction of the fracture. Scale bar = 200μm.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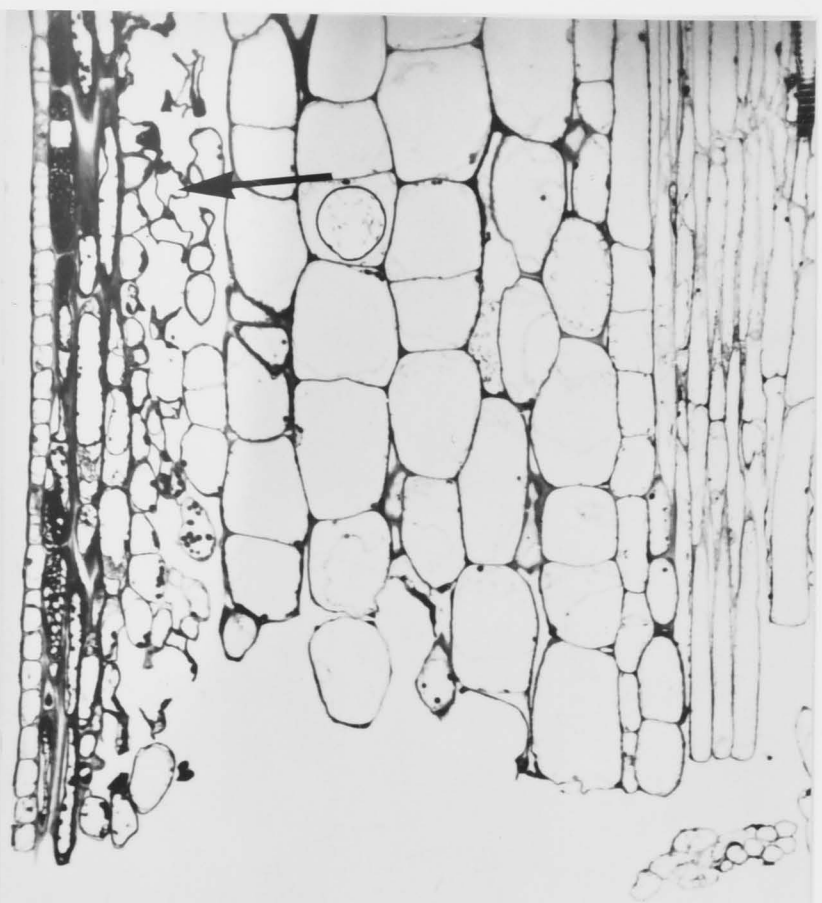

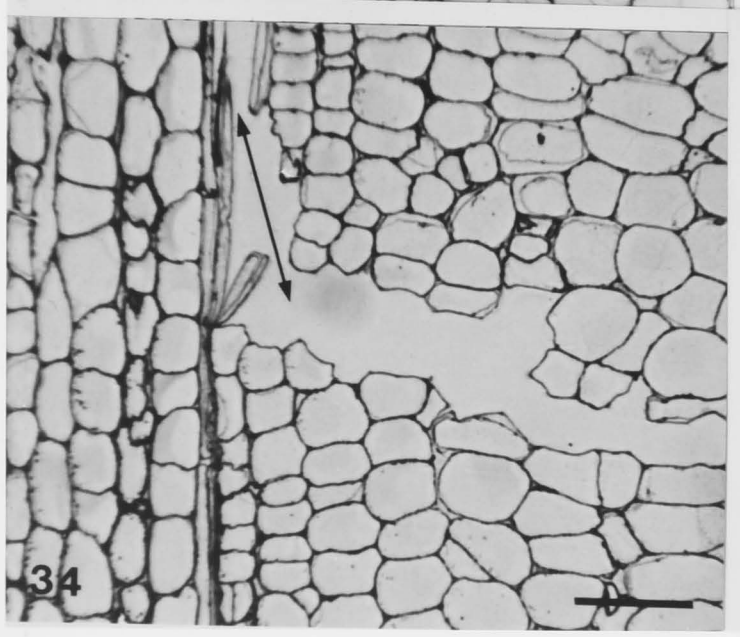
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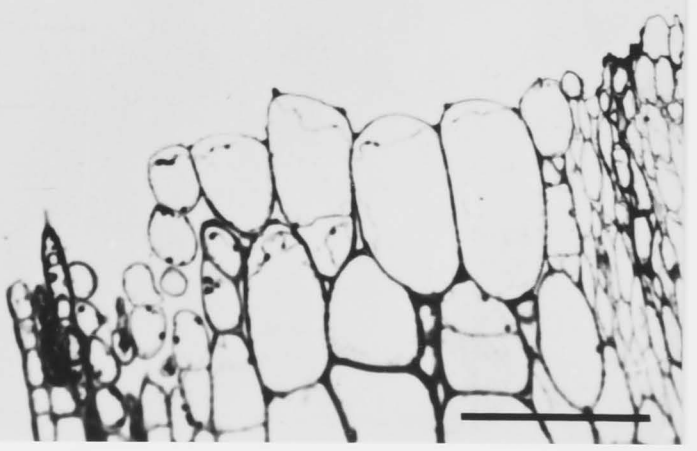

33

Figures 35-40. *Begonia corallina* (D = dictyosomes; large arrowhead = fracture surface).

Figs 35-36. Separation layer cells in the pith. Note the active dictyosomes (D) associated with numerous vesicles. Points of attachment before separation (Fig. 36.) are indicated by small arrowheads.

Scale bar = 2μm.

Fig. 37. An outer cortical cell at the proximal fracture surface after separation. Note the abundance of vesicles in the cytoplasm and microtubules (small arrowheads). The cell wall appears swollen and fenestrated (arrow).

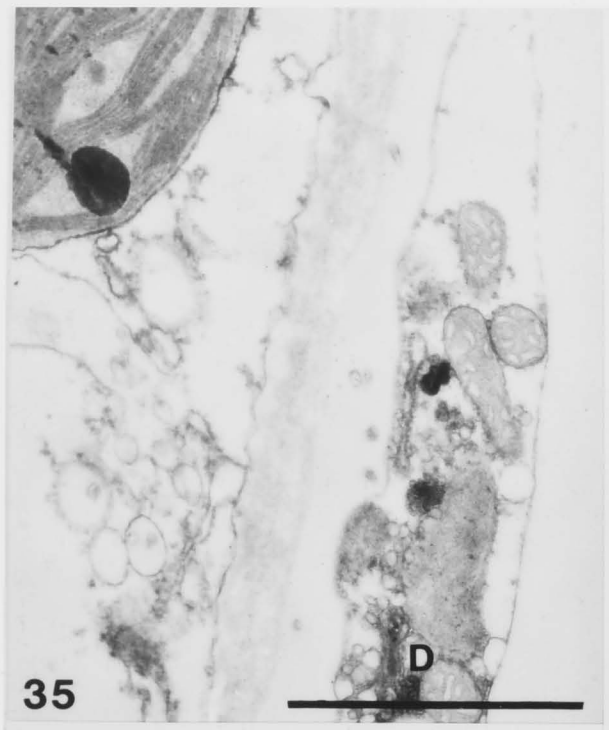
Scale bar = 5μm.

Fig. 38. Pith cells found 6 cell layers distal to the separation layer. Note the cell separation initiating at intercellular space and some vesicle-like material within inter cellular spaces (arrow).

Scale bar = 10μm.

Figs 39-40. An area of a completely separated cell in the pith after abscission. Note the dictyosomes associated with vesicles (Fig. 39.), convoluted plasmalemma (arrows) and vesicles fusing with the plasmalemma (Fig. 40, V). The cell wall is lined with dispersed microfibrils (DM). Both Fig. 39 and Fig. 40 are from the same cell.

Scale bar = 2μm.


D
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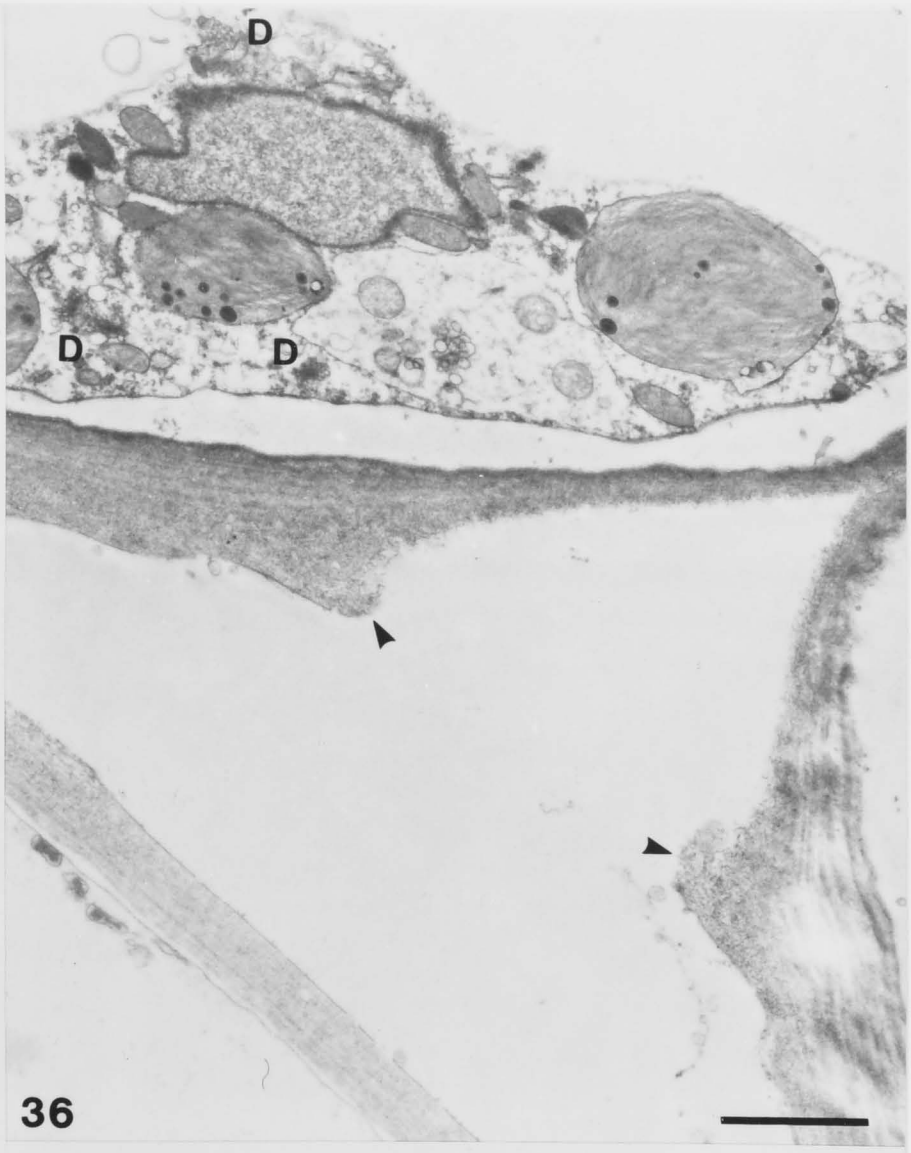
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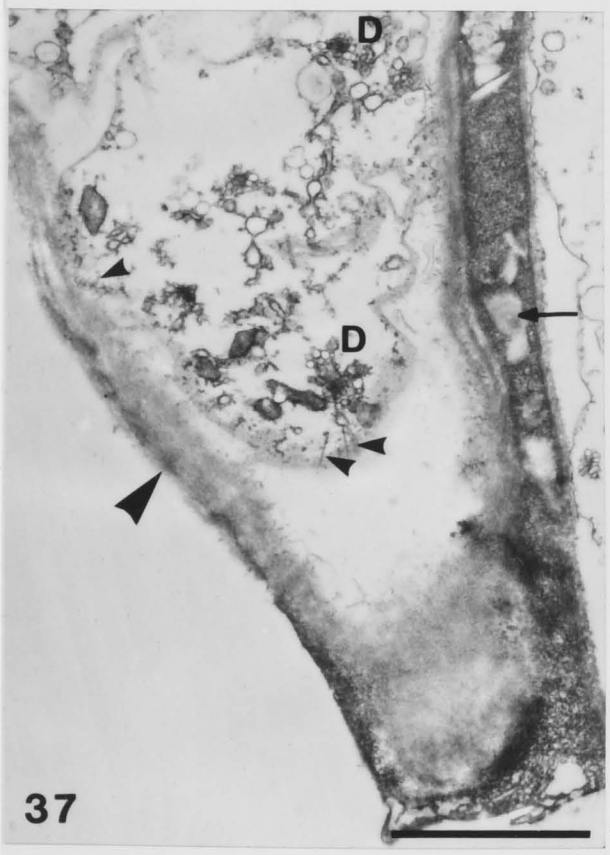
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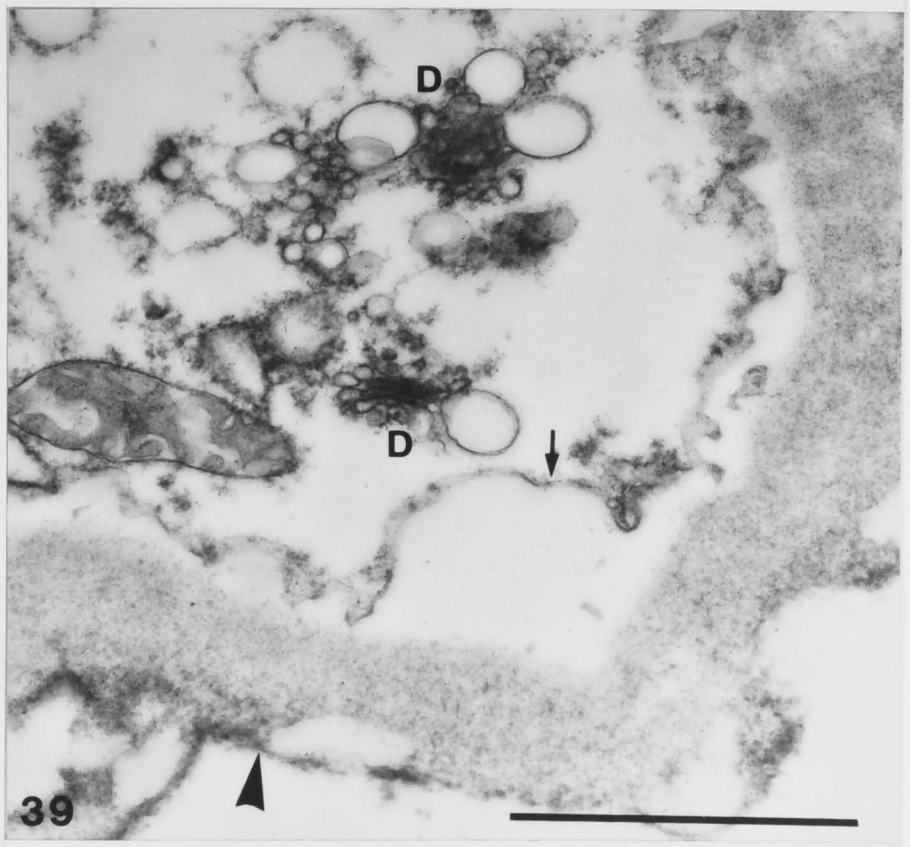
D
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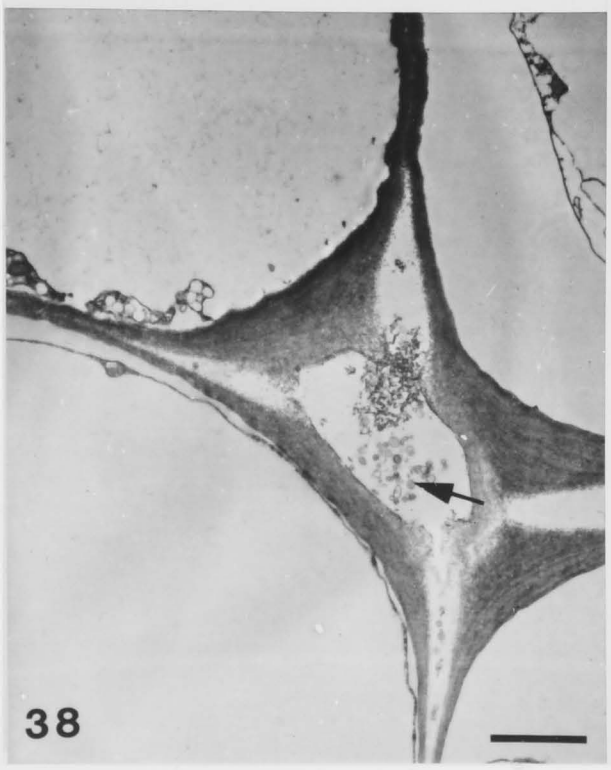
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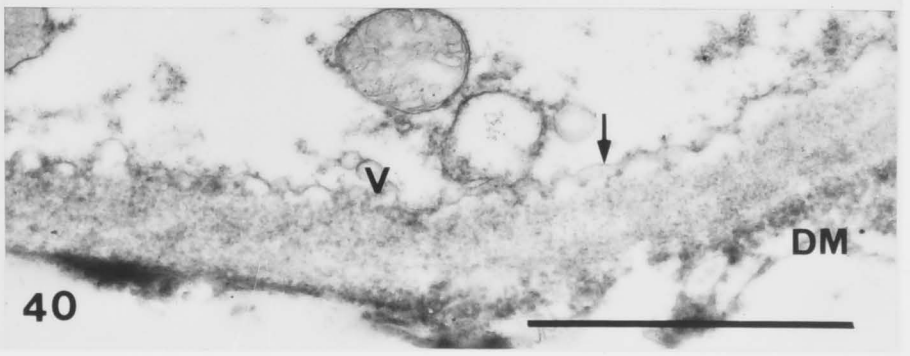
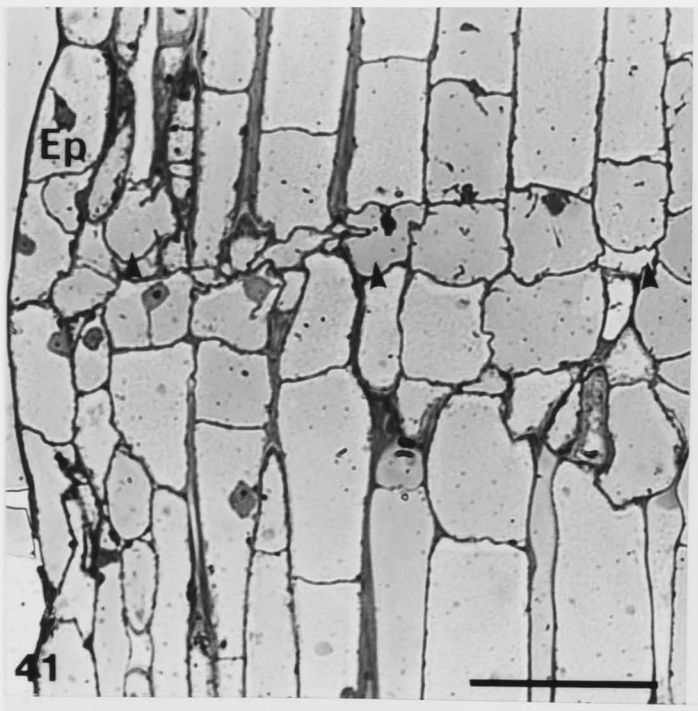

V
DM
40

Figures 41-48. *Ipomoea batatas*: light micrographs of longitudinal sections through the separation layer area.

Figs 41-42. Cell division in the cortex (Fig. 41) and pith (Fig. 42) at the onset of separation layer development (3 days after explanting). Arrowheads = newly divided cells. Scale bar = 50µm.

Fig. 43. The separation layer 4 days after explanting. Note the course of the separation layer, which moves downwards in the pith (arrow) and upwards in the vascular area (large arrowhead). Scale bar = 100µm.

Fig. 44. Xylem vessels with tyloses (Ty) found in close proximity to the separation layer (arrow). Pi = pith cells. Scale bar = 100µm.


Ep
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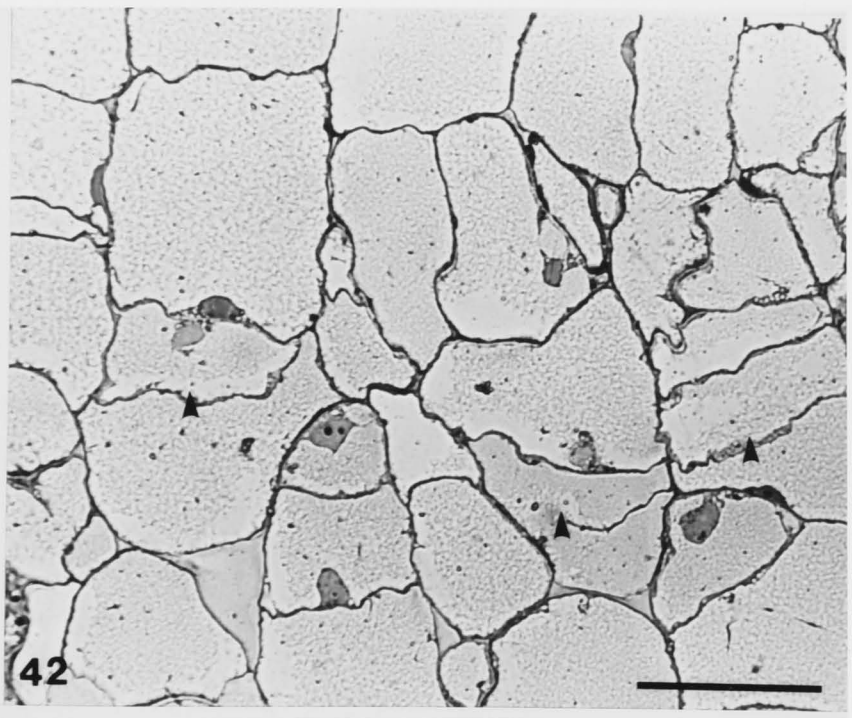
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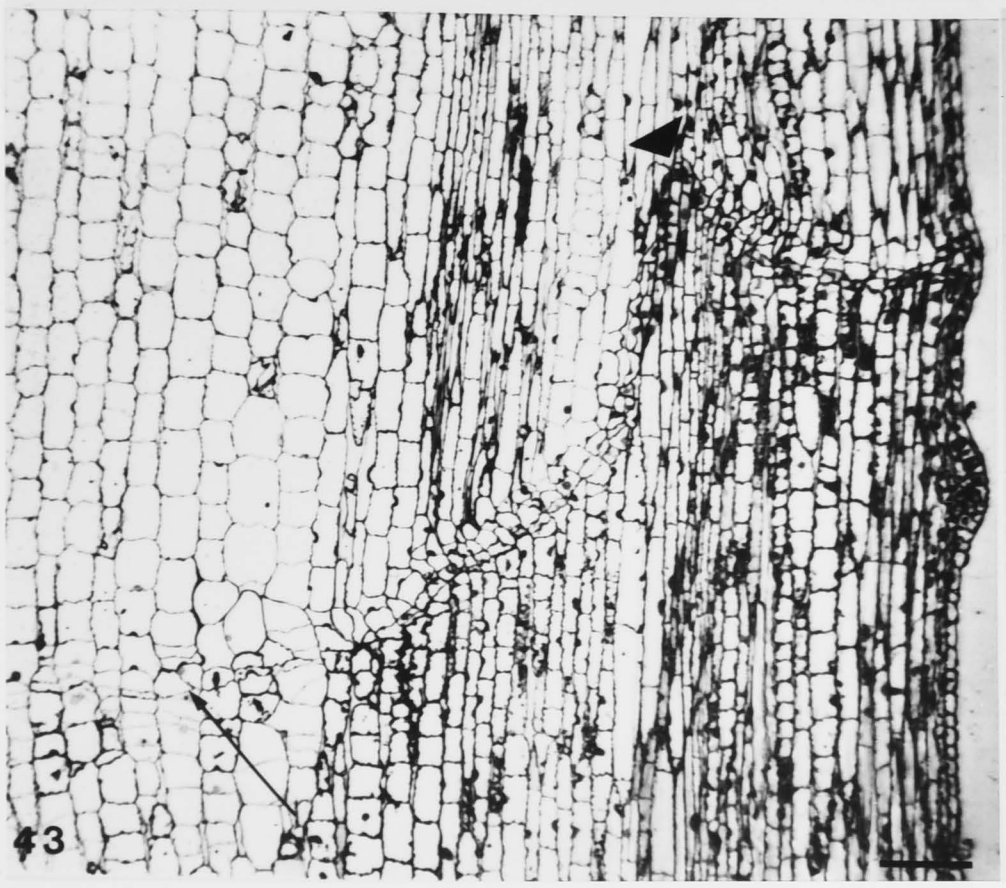
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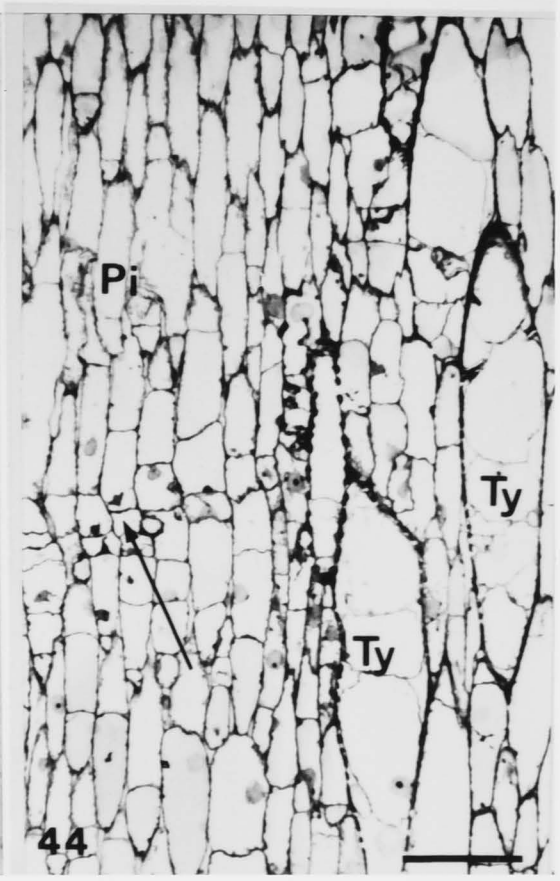
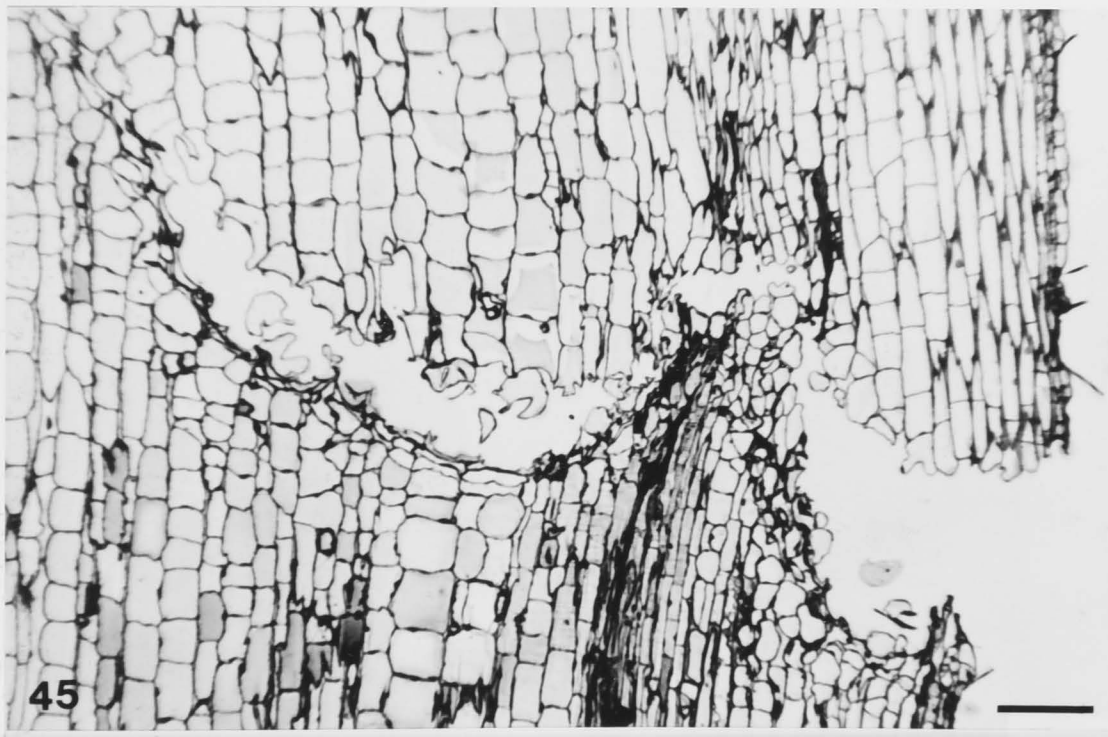

Pi
Ty
Ty
44

Figs 45-46. The upper separation layer, close to completion of separation. Note the confinement of divided cells to the lower segment. Cell division is also prominent in the cambial and phloem parenchymatous region (Fig. 46, arrow).

Scale bar = 100μm.

Figs 47-48. Longitudinal sections through ethylene induced separation layers. Note the enlarged cells (arrows) at the separation layer.

Scale bar = 100μm.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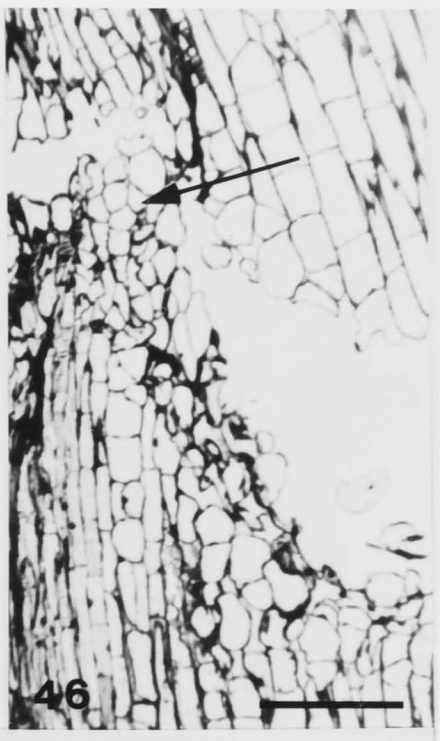
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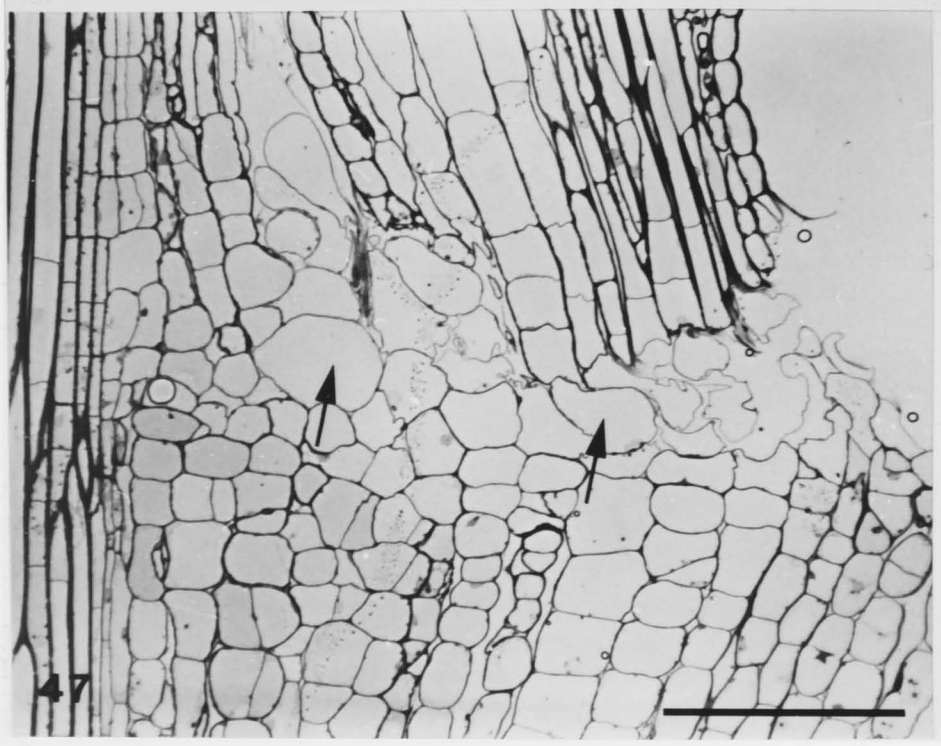
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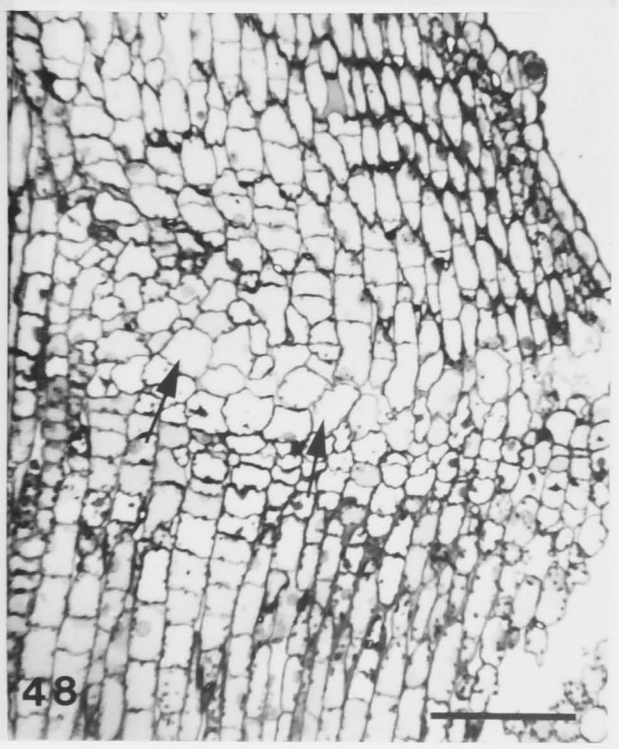

48

Fig. 49. *Ipomoea batatas*; electron micrograph of a cortical cell 8 cell layers below the separation layer (5 days after explanting). Note the absence of active dictyosomes. Scale bar = 5µm.

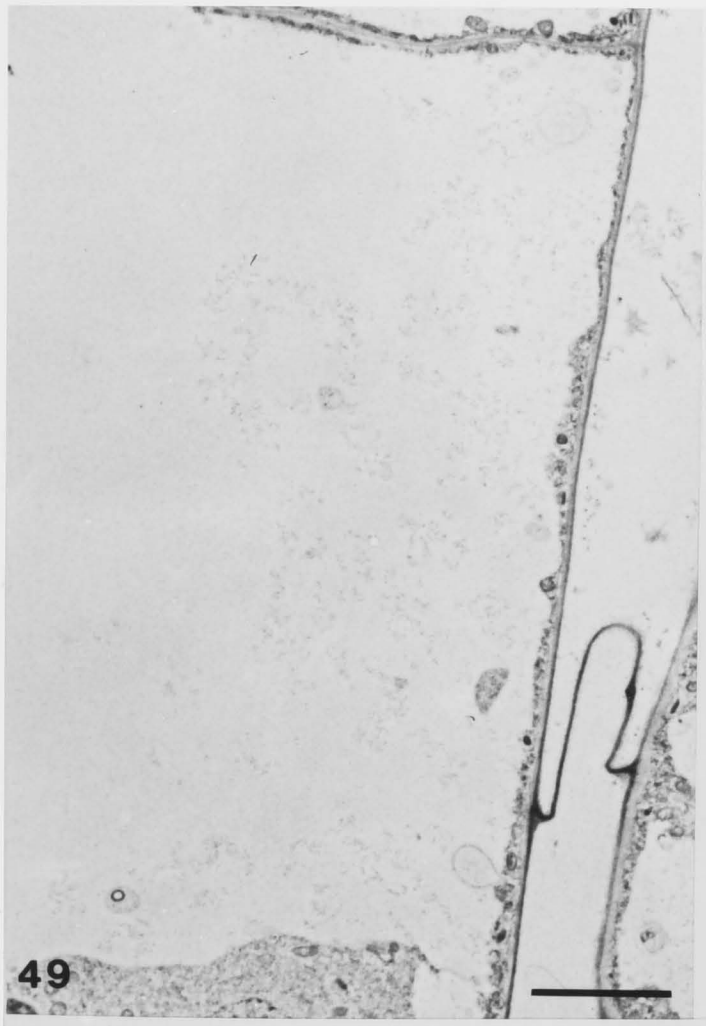
Figures 50-57. *Ipomoea batatas*: electron micrographs of separation layer area. (D= dictyosomes; RER = rough endoplasmic reticulum; SM = separating microfibrils).

Figs 50-51. Newly divided cells in the cortex at the onset of cell division at the separation layer (4 days after explanting). Note the abundance of dictyosomes and rough endoplasmic reticulum. Arrow = newly formed cell wall. Scale bar = 5µm.

Fig. 52-53. Inner cortical cells on the proximal side of the fracture plane at a later stage (5 days after explanting), than Fig. 50. Note very active dictyosomes, dilated RER (large arrowhead) and separating cells lined with separating microfibrils.

Fig. 52. Scale bar = 5µm.

Fig. 53. Scale bar = 2µm.



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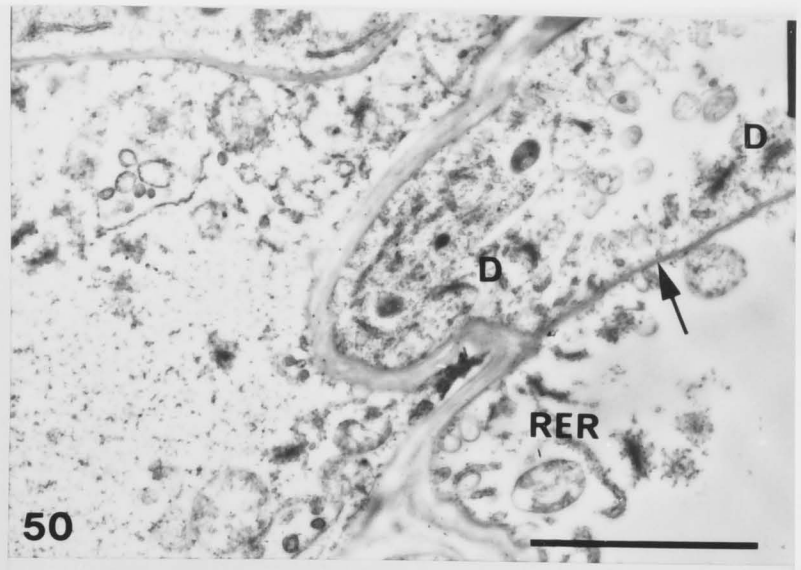

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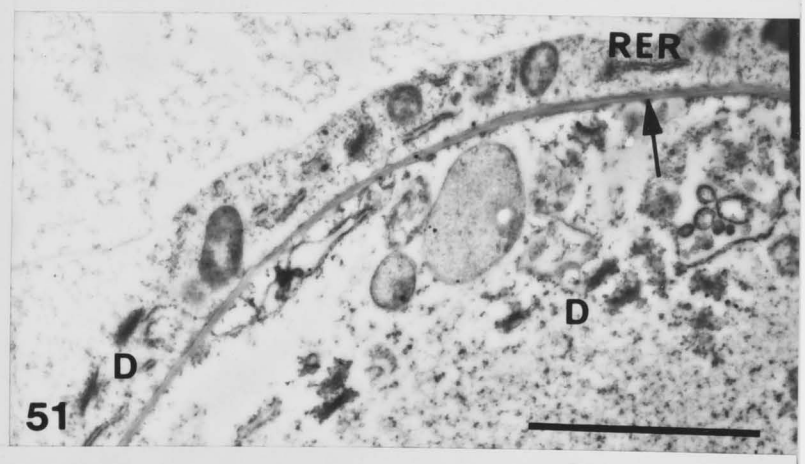

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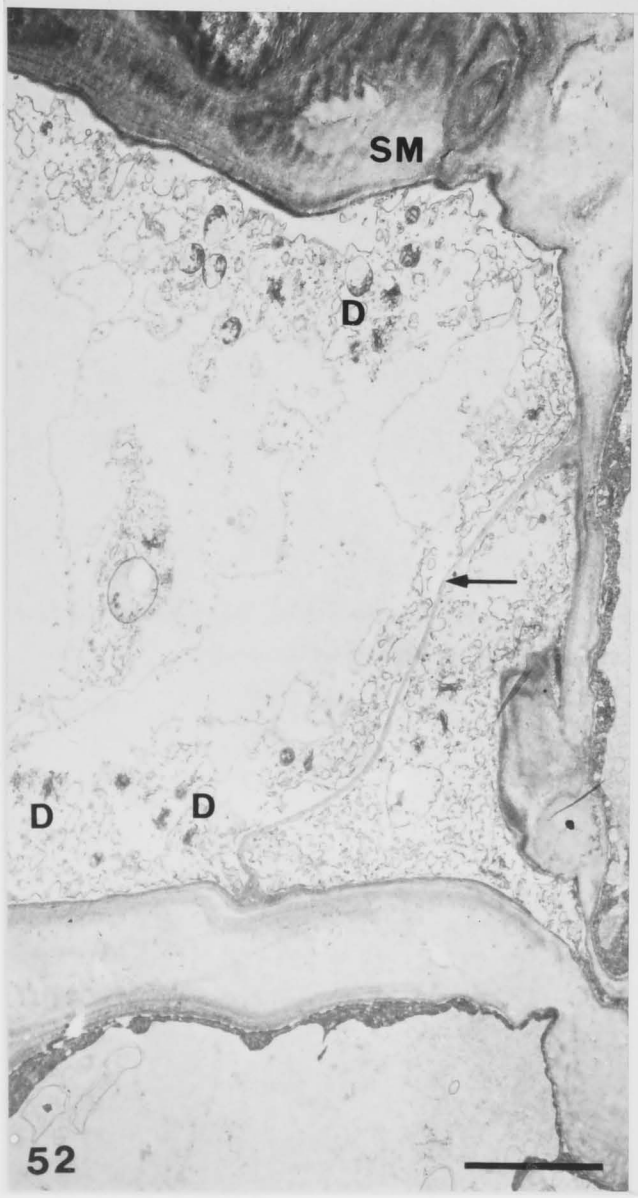

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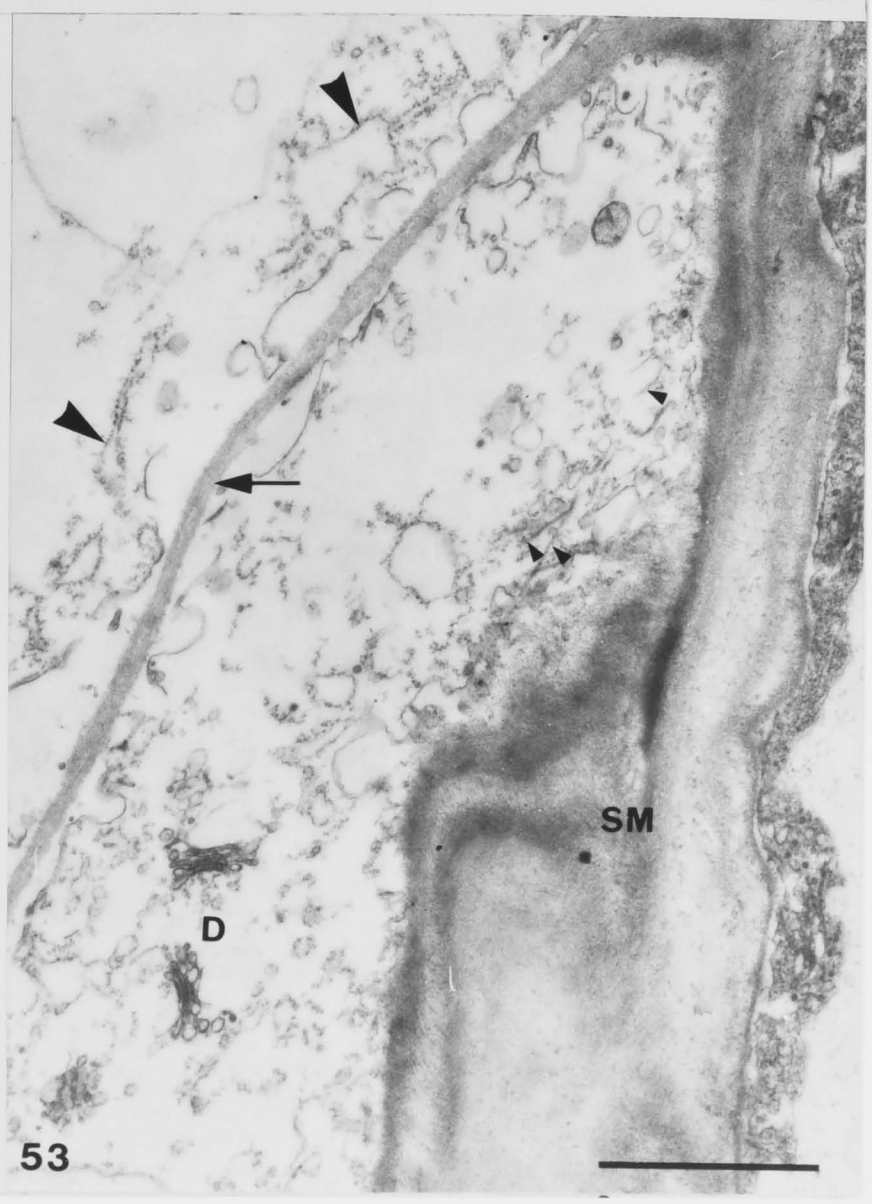


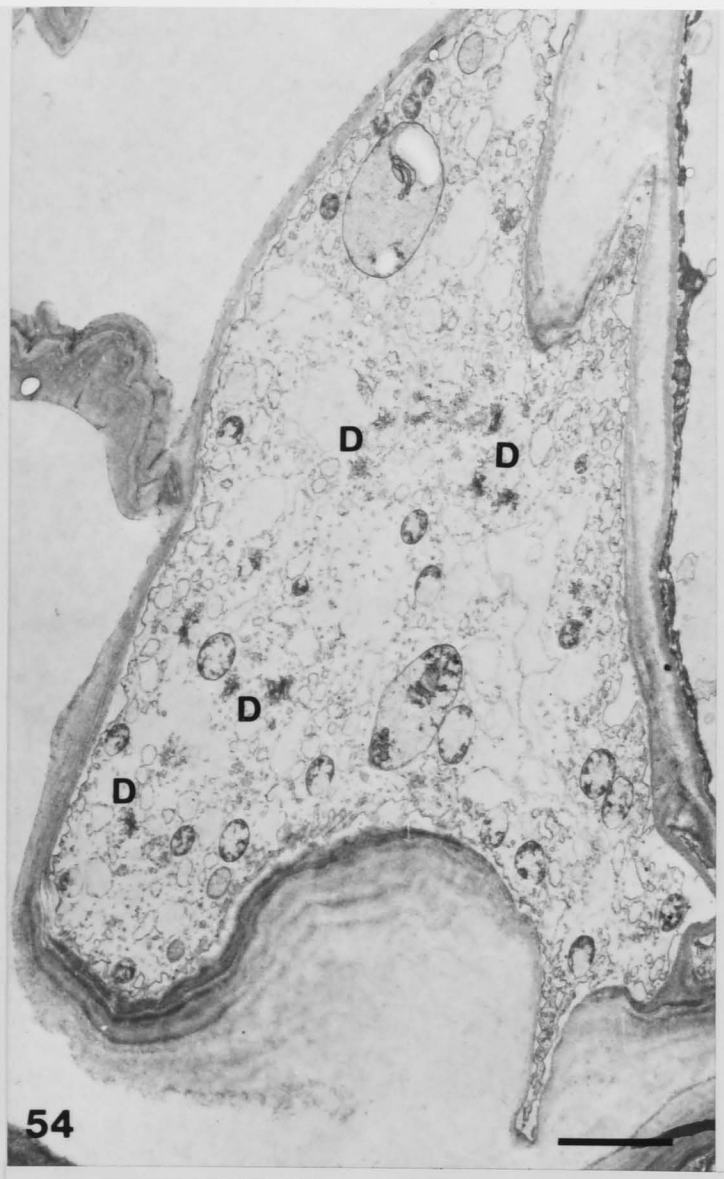
53

Figs 54-56. A separated cortical cell on the distal side of the fracture plane. Note the abundance of dictyosomes and cytoplasm with numerous dictyosomal vesicles (V). RER also appear dilated (Fig. 55, arrowhead). Plasmalemma appears highly convoluted (Figs 55-56), and microtubules are also prominent (Fig. 55, small arrowhead). Fig. 55 and 56 are enlargements of the cell in Fig. 54. Double headed arrow indicate the fracture line.

Scale bar = 3μm.

Fig. 57. A tylose (Ty) filled xylem vessel close to the separation layer.

Scale bar = 7μm.



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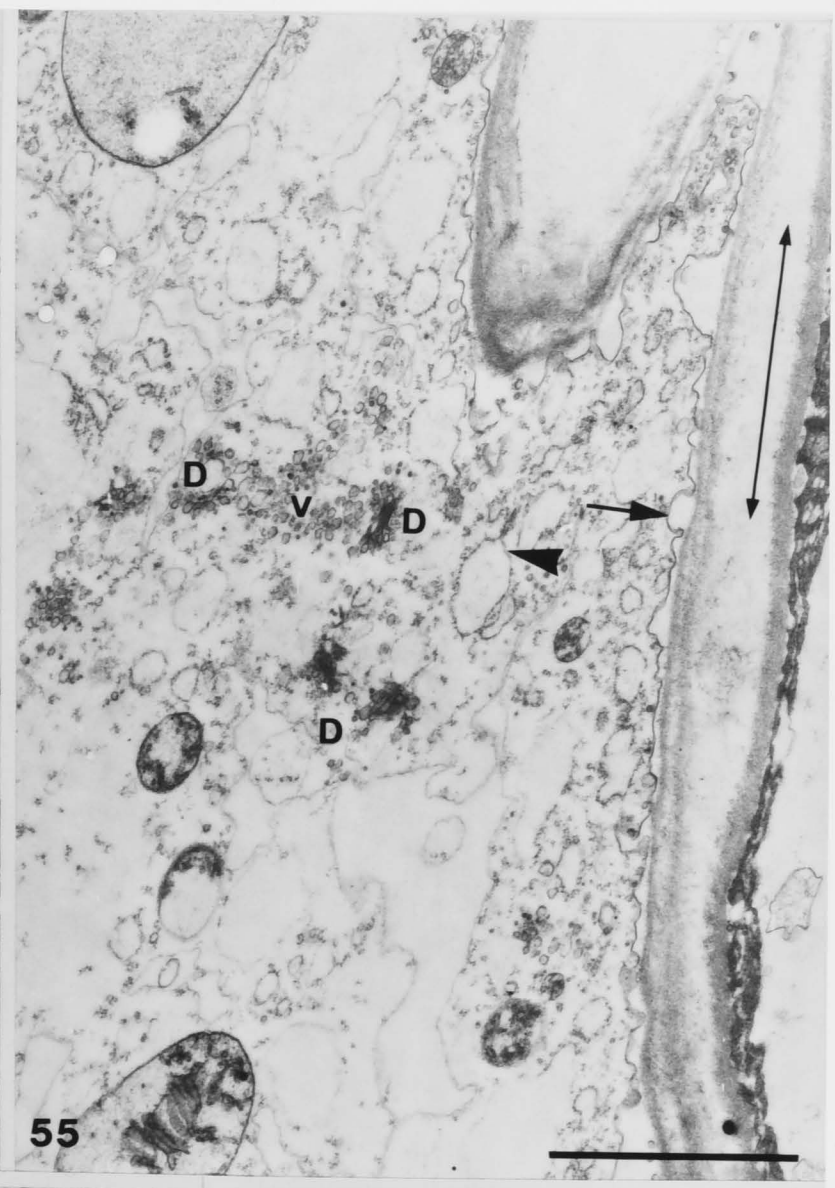

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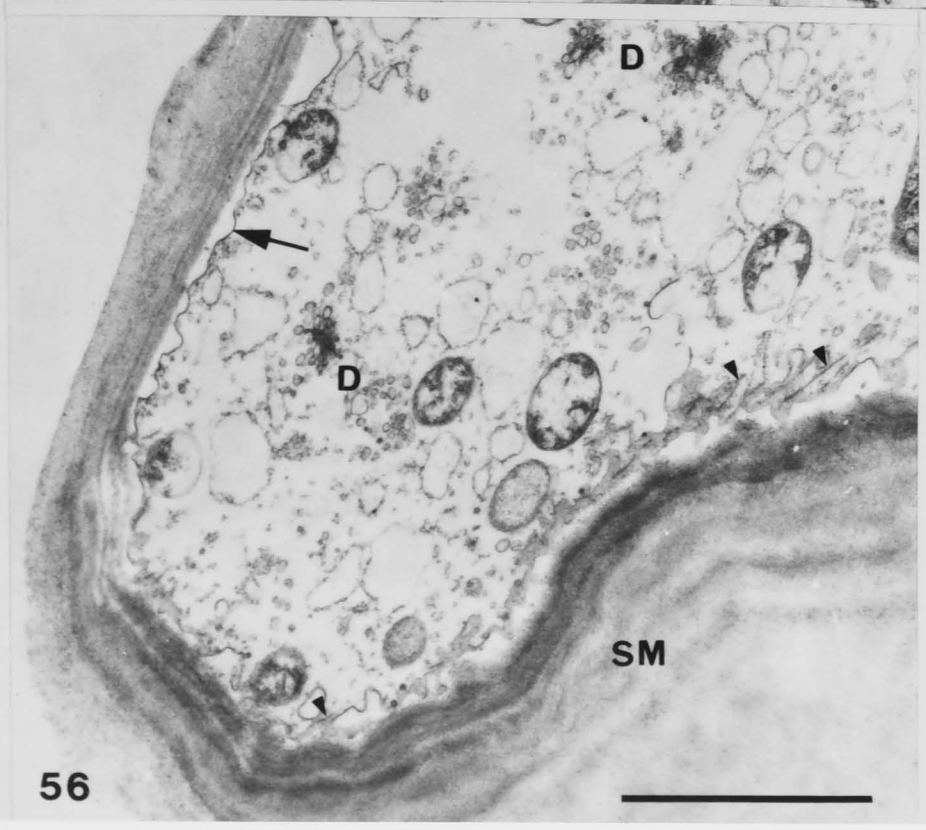

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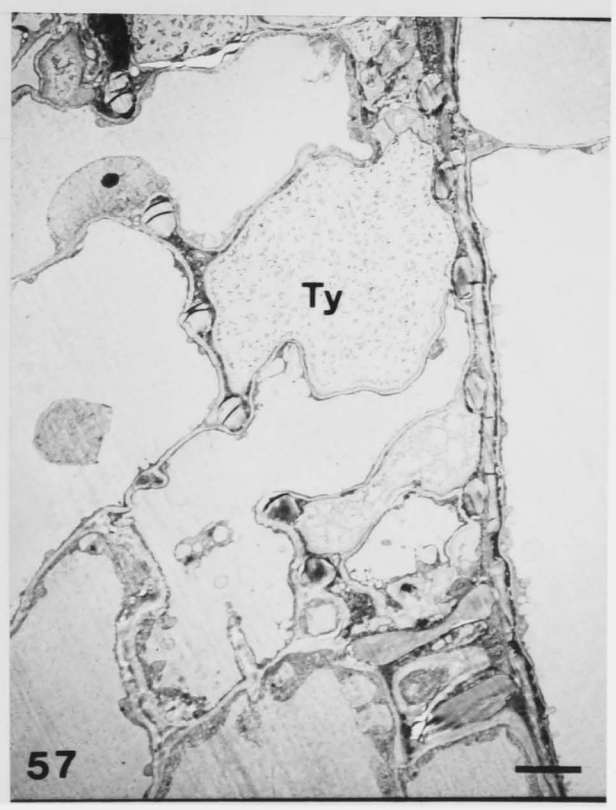

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Figures 58-62. Scanning electron micrographs of the proximal fracture surface after abscission.

Figs 58-59. Note the intact cells in the cortex (Fig. 59, Co). Some cells in the pith (Pi) appear fractured without separation (Fig. 59).

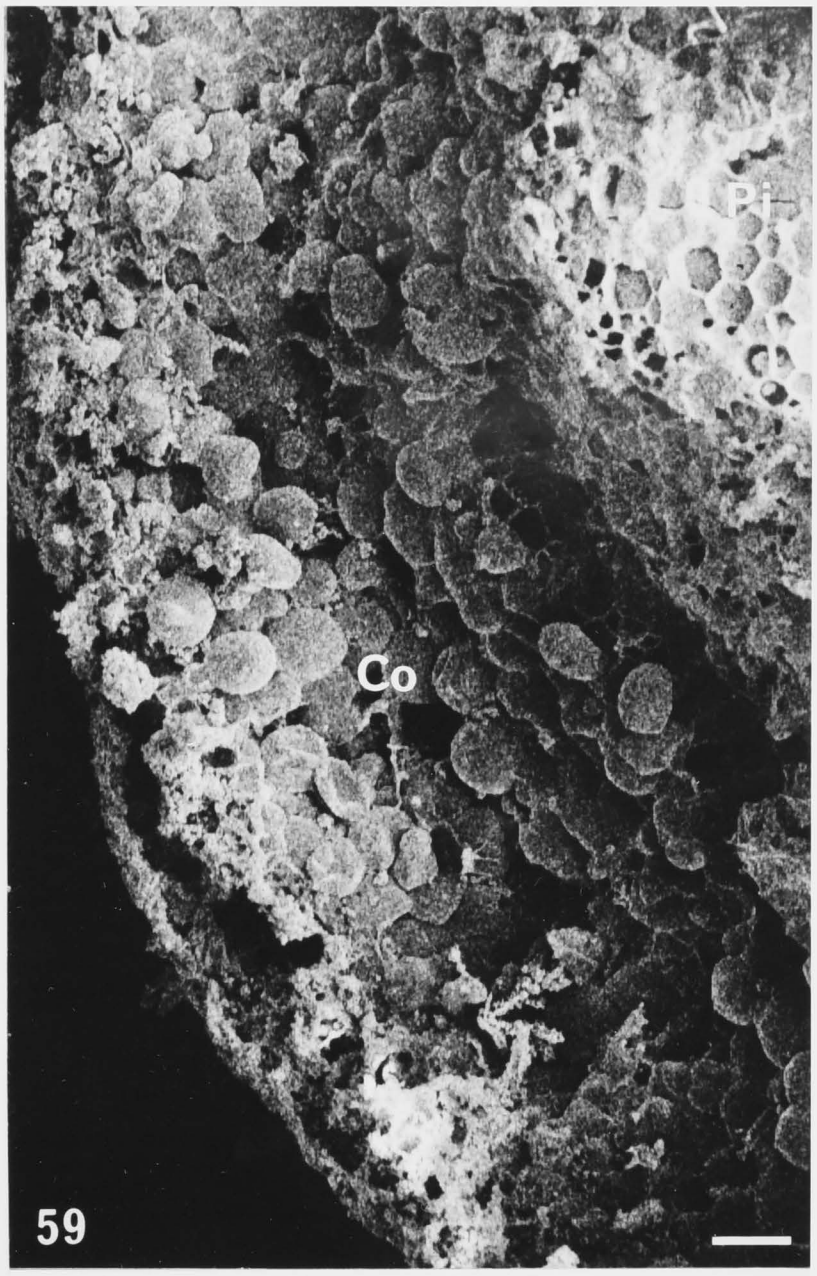
Fig. 58. Scale bar = 0.5mm

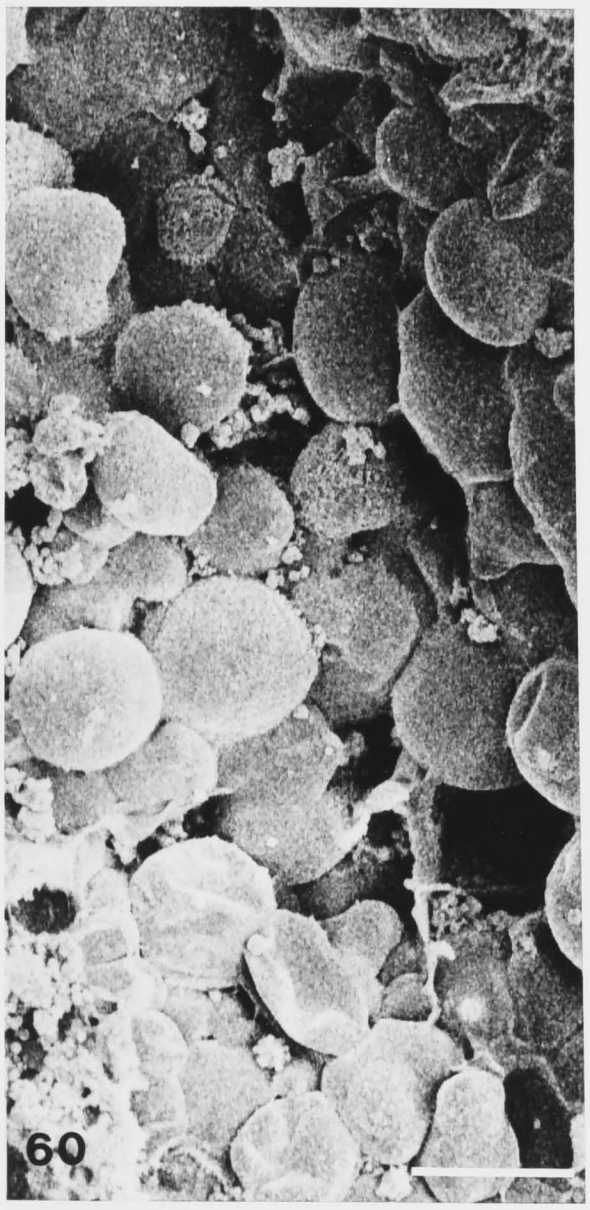
Fig. 59. Scale bar = 50µm.

Figs 60-62. Higher magnification micrographs of the cortical area of Fig. 59. Note the intact and turgid cells, dense deposits on the cells (Figs 61-62, arrowheads) and gelatinous material (Fig. 62, arrow). Also note the large granular deposits (G) associated with the outer cortical region (Figs 60, 62).

Scale bar = 50 µm.


58


Pi
Co
59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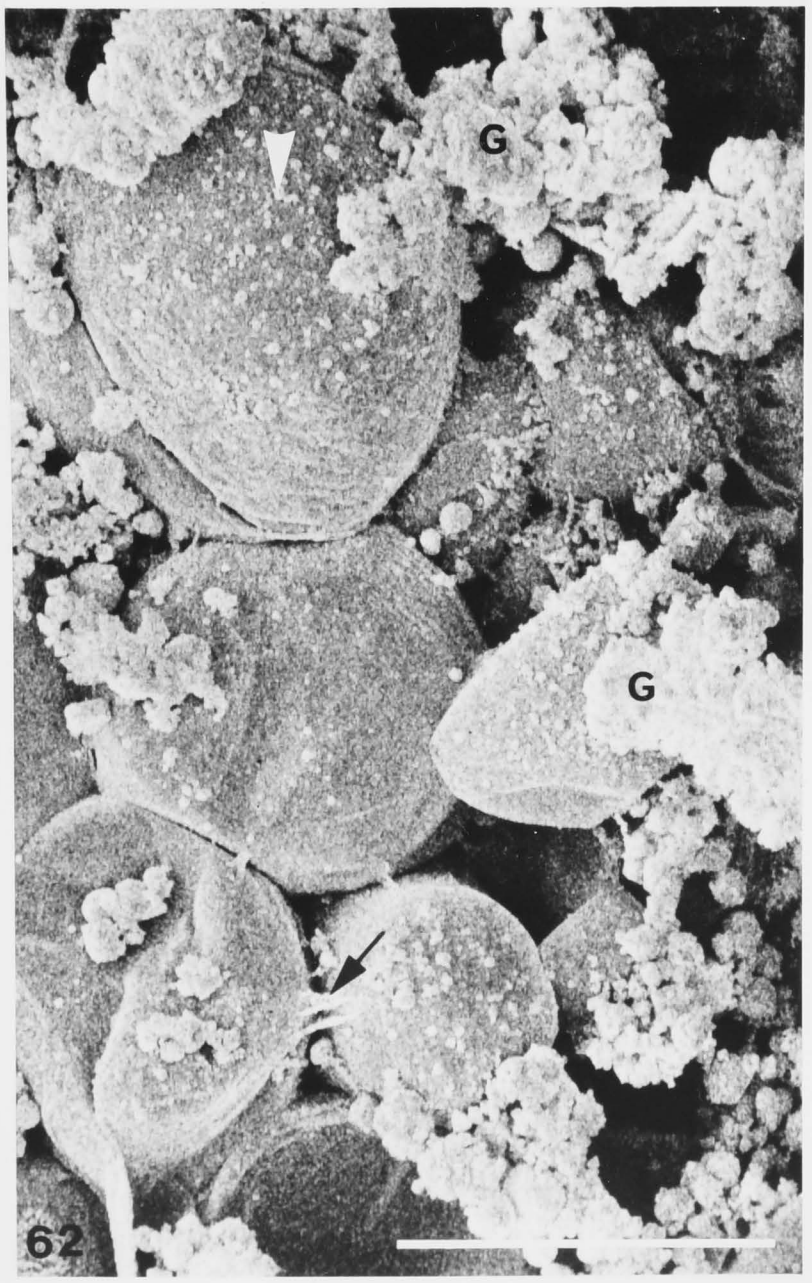
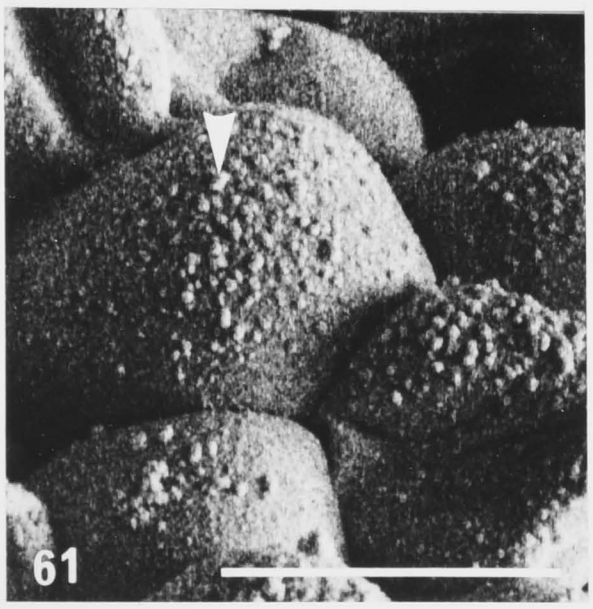

G
G
62


61

CHAPTER FOUR

Investigation of respiratory properties of

Impatiens sultani

internodal explants undergoing adventitious abscission

1. INTRODUCTION

Impatiens sultani internodal explants undergoing adventitious abscission sometimes show signs of senescence such as yellowing of the segment above the separation layer. This is somewhat comparable to the abscission of intact leaves or fruits where the yellowing of the leaf blade or the fruit usually precedes abscission.

During senescence of detached leaves a significant respiratory burst is often observed (Tetley and Thimann 1974), similar to the climacteric respiratory peak which accompanies the ripening of many fruits (Solomos and Laties 1974b; Biale and Young 1981). Even leaves attached to the plant have been reported to exhibit doubling of respiration just before abscission (Hardwick *et al.* 1968). However, there are some reports where the respiratory rate drops gradually during senescence (Smillie 1962; Richardson 1957).

Involvement of respiration in abscission has been demonstrated by its dependence on O_2 (Addicott 1982), and Carns (1951) demonstrated that inhibitors of respiratory enzymes, including cyanide, were able to inhibit abscission. Carns (1951) also found that abscising explants produced a 'climacteric' like rise in respiration. Marynick (1977) observed that during abscission of cotton explants, the region close to the abscission zone had a higher rate of respiration than other regions of the explant.

The climacteric-like increase in leaf respiration has been attributed sometimes to uncoupling of oxidative phosphorylation (Millerd *et al.* 1953; Thimann *et al.* 1977) and also to an increase in cyanide resistant respiration (alternative oxidase; Solomos

and Laties 1974a, 1975). Later, Theologis and Laties (1978) reported that in avocado slices, the climacteric rise involved both the alternative and cytochrome pathways.

Many plant tissues exhibit this cyanide-insensitive, non-phosphorylating oxidative system that branches from the respiratory chain at the level of ubiquinone (Day *et al.* 1980; Laties 1982; Siedow and Berthold 1986), in addition to the main cytochrome chain (Fig. 1). Substituted hydroxamic acids have been widely used as specific inhibitors of the alternative oxidases (Schonbaum *et al.* 1971; Azcon Bieto *et al.* 1983). The alternative path is only engaged when the cytochrome path is saturated or when it is inhibited (Day *et al.* 1980). Saturation can occur at low ADP:ATP ratios and high levels of substrate availability in the cell (Laties 1982). It can also occur if electron transport is uncoupled, by adding carbonyl cyanide p-trifluromethoxy phenyl hydrazone (FCCP) or carbonyl cyanide m-chlorophenyl hydrazone (CCCP), providing substrate availability is not limiting.

In the present study the nature of respiratory control of the upper, senescing, and basal, seemingly non-senescing, parts of explants undergoing adventitious abscission were investigated with the use of inhibitors (KCN and SHAM: salicylhydroxyamic acid) and an uncoupler (CCCP). The changes in soluble sugars within the explants during abscission were also investigated.

2. MATERIALS AND METHODS

2.1 Plant Material and Culture

Internodal explants (20mm in length) obtained from *Impatiens sultani* plants were cultured in the following media as described in Chapter 1, and incubated at 24^{o} C in the dark.

(1) 0.0001% IAA + 1.0% sucrose

(2) 0.0001% IAA + 0.0001% zeatin + 1.0% sucrose

(3) 1.0% sucrose (control)

2.2. Respiration Measurements

After washing the base of the explants in distilled water to remove any adhering sucrose, explants were divided into 4 segments, each 5 mm in length. Each segment was cut into 0.5mm slices transversely with a razor blade in 20mM HEPES or TES buffer (pH 6.5). Two to three explants were used for each measurement. After incubating the tissue slices in the buffer for 25-30 minutes at room temperature in the dark, O_2 uptake was measured at 25 ^{o}C using an oxygen electrode (Rank Bros. U.K.) in 4 ml of the same buffer which was initially in equilibrium with air. A plastic grid was used to separate the stirrer of the electrode from the tissue slices. Aliquots of respiratory effectors, SHAM (1M in 2-methoxyethanol), KCN (0.1M in water), CCCP (0.05M in ethanol) were added directly into the reaction vessel from the respective stock solutions.

2.3. Cytochrome Assay

At regular intervals during incubation, explants were divided into 4 segments of 5mm length, after washing the basal part of the explant with distilled water. The vials containing the different segments were immediately frozen in liquid N_2 and stored in a -70^{o}C freezer until analysis.

The frozen tissues were ground in 2 ml of mitochondrial reaction medium on ice and sonicated (100W, 30sec, using a Labsonic 1510 sonicator). The homogenate was centrifuged (at 14,250xg for 3 minutes, using a Beckman microfuge) to remove suspended tissue fragments. An appropriate volume of the supernatant was used to assay the cytochrome c oxidase activity based on the oxidation of reduced cytochrome c (ferrocytochrome c) followed as a decrease in absorbance at 550 nm (Smith 1955).

'Reduced cytochrome c' was prepared by reducing a solution of cytochrome c (15mg/l) with ascorbic acid and subsequently dialysed for 24 hours (against 3 changes of deionized water) to remove ascorbate. 20 µl of this reduced cytochrome c was added to 0.9 ml of mitochondrial reaction medium. After recording the initial absorbance at 550 nm (using a Pye Unicam 8800 uv/vis. spectrophotometer), an appropriate volume of the extract was introduced and the change in absorbance at 550nm recorded for a few minutes. Subsequently, 10 µl of 0.1M $K_3Fe(CN)_6$ was added to fully oxidize the cytochrome c, to determine the absorbance due to ferricytochrome c. The cytochrome c oxidase activity is expressed as µM cytochrome c oxidized min^{-1} g^{-1} f.wt. of tissue.

2.4. Analysis of Sugar

Explants were sampled as described in the cytochrome assay. The samples, after freezing in liquid N_2 were freeze-dried (using a Virtis 10-145 MR-BA freeze drier) for 24 hours, and stored at -20^{o}C until analysis. The tissues were ground in liquid N_2. 2ml of hot (95^{o}C) 10mM TES buffer (pH 7.5) was added to the samples and the homogenate was incubated in boiling water 5 minutes. After sonication (using a Bransonic 220 sonicating bath) the suspended tissue fragments were removed as a pellet by centrifugation (at 14,250 g for 3 minutes using a Beckman microfuge).

Soluble sugars were analysed using a glucose specific enzymic method (Bergmeyer *et al.* 1974), using glucose S. V. R. (Calbiochem Behring). Fructose was measured after converting to glucose with p-glucoisomerase (Sigma Chemical Company). Sucrose was hydrolysed to fructose and glucose by invertase (Sigma Chemical Company), at 37^{o}C for 2 hours.

For the glucose assay, glucose was converted to glucose 6-P by hexokinase, and then oxidized to 6-phosphogluconate by glucose 6-P-dehydrogenase, reducing a molar equivalent of NADP. The change in absorbance at 340nm (proportional to the glucose concentration from 0-10 mg/ml) was recorded using a Pye Unicam 8800 uv/vis. spectrophotometer.

2.5. Electron Microscopy

Tissue segments from the upper segment (5mm below the upper end of the explants) and the basal segment (1.5-2.0mm above the basal end) were fixed at different times during incubation and ultra thin sections were observed electron microscopically as described in Chapter 3.

3. RESULTS

3.1 Analysis of Respiratory Data

Figure 2 illustrates a simplified scheme of respiration and its control points. The control points could be in the availability of substrates for glycolysis, glycolysis itself, the TCA cycle or the electron transport pathways (flavoprotein, cytochrome and alternative). In each pathway of electron transport a distinction between activity (actual electron flow, v) and capacity (maximum possible electron flow, V) could be established by the use of specific inhibitors (Fig. 1) and uncouplers.

Electrons flowing through the flavoprotein pathway are distributed between alternative and cytochrome pathways. The non-phosphorylating alternative path usually operates only when the cytochrome pathway is saturated or restricted (Bahr and Bonner 1973; Theologis and Laties 1978).

The activity of the cytochrome pathway (v_{cyt}) is estimated by substracting the residual respiration (v_{res}, O_2 uptake insensitive to SHAM and KCN) from respiration resistant to SHAM (an inhibitor of alternative oxidase). The SHAM sensitive component represents the activity of alternative oxidase (v_{alt}). Respiration

in the presence of KCN minus residual respiration, represents the capacity of the alternative oxidase (V_{alt}).

Uncouplers, being protonphores, uncouple the flux of electrons through the cytochrome pathway from phosphorylation of ADP, thus reducing the electrochemical proton gradient across the inner mitochondrial membrane. This allows maximum electron transport via the cytochrome pathway which enables the capacity of the cytochrome pathway to be determined. Uncoupling could, however, reduce the cytosolic ATP:ADP ratio which can lead to enhanced glycolytic activity and increased flux of substrates through glycolysis in vivo (Fig. 2: site 3). This, in turn would lead to faster TCA cycle turnover and increased NADH availability for electron transport. This could lead to increased engagement of the alternative path. Thus the capacity of the cytochrome pathway is estimated by the O_2 uptake in response to an uncoupler (CCCP) and a substrate in the presence of an inhibitor of alternative path (viz. SHAM).

3.2. Preliminary Experiments

20 mm explants cultured in abscission inducing media (0.0001% IAA + 1% sucrose and 0.0001% IAA + 0.0001% zeatin + 1.0% sucrose) were sampled at different times after explanting. These were divided into 4, 5mm segments and the rate of O_2 uptake by each segment measured Table 1). Two to three explants had to be used for each measurement to obtain measurable rates of O_2 uptake, especially during the later stages of abscission. The results showed that the rate of O_2 uptake declined in an acropetal direction in the explant, throughout the incubation period

(Table 1), with the upper two segments (each 5mm long) showing the lowest rate. The third segment (from the upper end), containing the future separation layer showed intermediate values between that of the upper segment and the basal segment. This segment consisted of a significant proportion of the tissue above the separation layer which had lower rates of respiration. Therefore, in most of the subsequent experiments, this segment was avoided and only the basal 5mm segment (designated as no. 4 segment) and the upper two segments (no.1 and no. 2) were used.

To determine the optimum concentration of SHAM to be used as an inhibitor of the alternative oxidase, 6 concentrations of SHAM (1, 2, 3, 4, 5, and 6 mM) were used, in the presence of 0.3mM KCN (which inhibits cytochrome oxidase). From this, the optimum concentration of SHAM to be used to inhibit alternative oxidase was found to be within 4 to 5 mM (Fig. 3).

3.3. Effect of KCN and SHAM

3.3.1. Upper segments

The upper segments (segment no.1 and no.2) of explants cultured in abscission inducing media (IAA + zeatin or IAA), demonstrated a distinct drop in the rate of O_2 uptake during incubation (Fig. 4). Although the same drop was observed in the non-abscising explants in the control media, the respiration of the latter remained significantly higher than that of the abscising segments throughout incubation (Fig. 4). SHAM did not inhibit the O_2 uptake at any stage of incubation, indicating that the alternative path was not engaged at any stage (activity of the alternative pathway,

$v_{alt} = 0$). The drop in the respiratory rate therefore appeared to be due to a progressive reduction in the cytochrome path activity (Fig. 5). The capacity of the alternative pathway (estimated by KCN resistant O_2 uptake), also showed a similar decline during incubation (Fig. 6).

3.3.2. Basal segment

The basal segments, by contrast, showed an increase in their O_2 uptake rate (after an initial slight drop), just before abscission (i.e. on the 4^{th} day in IAA only media and on the 3^{rd} day in IAA + zeatin media). After abscission, the rate of O_2 uptake decreased gradually (Fig. 7). Since at no stage was there any engagement of the alternative path, and since residual respiration did not change significantly, the observed increase in O_2 uptake in Fig. 7 was due to an increase in the cytochrome path activity (Fig. 8). Although alternative oxidases were not engaged at any stage, the capacity of alternative path showed a marked increase before abscission and a subsequent decrease (Fig. 9).

The significant increase in the cytochrome path activity and the capacity of the alternative oxidase was not observed in non-abscising explants in control media.

3.4. Effects of Substrates and Uncoupler

Tables 2 and 3 show the results of adding substrates and uncoupler to upper and basal segments on the rate of O_2 uptake at 2 stages of incubation (day 3 and 5).

3.4.1. Upper segments

The substrates, sucrose (Table 2, Exp. 2) and malate (Table 2, Exp. 1) were only able to stimulate the rate of O_2 uptake by 8-10%, in the upper segment of abscising explants, on day 3 of incubation. This indicated that the drop in respiration was not due to limitation of respiratory substrates. In non-abscising controls, however, substrates sucrose (Table 2, Exp. 2) and malate (Table 2, Exp. 1) were able to stimulate O_2 uptake considerably (25% stimulation by sucrose and 35% by malate).

Uncoupler was able to stimulate O_2 uptake in both abscising and control explants to a greater extent (Table 2, Exp. 3) with a 60-100% stimulation being observed. Subsequent addition of malate significantly increased the rate of O_2 uptake only in non-abscising control explants. A significant proportion of O_2 uptake after substrate and uncoupler addition was due to the engagement of the alternative oxidase in the day 3 segments of both abscising and control explants (demonstrated by the inhibitive effect of SHAM: Table 2, Exp. 1,2,3).

The capacity of the cytochrome pathway (indicated by O_2 uptake after substrate, uncoupler and SHAM addition) diminished during incubation when compared to the explant pre-culture. This drop in the capacity of the cytochrome pathway was more pronounced in abscising explants than in the controls (Table 2, Exp. 1).

By day 5 the apical segments of abscising explants could not be stimulated significantly even with the addition of both substrates and the uncoupler (Table 2, Exp. 1). The control explants however could be stimulated by uncoupler and substrate to greater extent even on day 5 (Table 2, Exp. 1).

3.4.2. Basal segment

Basal segments of abscising and control explants, being in contact with 1% sucrose in the medium during incubation, did not show a significant increase in the rate of O_2 uptake in response to the substrates sucrose or malate (Table 3, Exp. 2,1). Uncoupler alone (or with substrate) was able to stimulate O_2 uptake to a greater extent in day 3 basal segments (Table 3, Exp. 1,2), than in the explant pre-culture (Table 2, Exp. 1,2). The abscising explants showing a slightly higher stimulation than the control explants. This increase in O_2 uptake in response to uncoupler was due to increased participation of the alternative oxidase (shown by the greater inhibition by SHAM), when compared to the values for explant pre-culture (Table 2, Exp. 2; Table 3, Exp. 2). This corresponded to the increase in the capacity of the alternative pathway in abscising segments by day 3-4 of incubation (Fig. 9).

The capacity of the cytochrome pathway, on the other hand, remained approximately constant between explant pre-culture, day 3, and day 5 basal segments (Table 2, Exp. 1; Table 3, Exp. 1). The non-abscising controls however showed a marginally lower capacity on day 5 (Table 3 Exp. 1).

3.5. Cytochrome Oxidase Activity

Both upper and lower segments of explants demonstrated similar extractable cytochrome oxidase activity during incubation (Table 4). The activity increased gradually up to the 3rd day of incubation and then decreased. Explants in abscission inducing medium (IAA + zeatin) and control medium did not exhibit a significant difference between them in the activity during incubation.

3.6. Tissue Sugar Concentrations

The sugar content of explant segments, measured at different stages of incubation are shown in Table 5. Although glucose, fructose and sucrose were analysed separately, their ratio was fairly constant at most stages (as reported for nodal abscission, Warren Wilson *et al.* 1987), The basal segments of explants, however, demonstrated an increase in the content of sucrose during the later stages of incubation. In Table 5 only the total sugar content are presented.

The results show that there was a gradual decrease in the tissue sugar content in the apical parts of the explant in both abscising and control explants. However, in abscising explants (in both IAA and IAA + zeatin media) this decrease was significantly faster than in control explants.

Basal segments on the other hand maintained a high concentration of sugar throughout incubation with a tendency to increase with time, this increase being more prominent in abscising explants than in controls.

3.7. Ultrastructural Observations

During incubation, the upper segment of abscising explants demonstrated signs of senescence (on day 3), such as reduction in ribosomes and decrease in cytoplasmic density. The mitochondria started to show signs of disintegration only by day 5 of incubation (Figs 10a-10c). At this stage, mitochondria could be seen in different stages of breakdown (Figs 10b-10c).

The cells of the basal segment, in contrast, maintained a denser, seemingly more active cytoplasm with abundant ribosomes and endoplasmic reticulum (Fig. 11a,c,

12), although some cells showed increasing numbers of vesicles during later stages of incubation (Fig 13). The mitochondria also appeared intact (Fig. 11b, 12).

Some cortical cells also showed a high number of mitochondria (Fig. 11a). Although this increase was very much more marked when compared to that of the upper segment. Whether it is significantly higher than that of the controls or from initial, cannot be determined conclusively without recourse to more elaborate methods.

4. DISCUSSION

4.1. Respiratory Regulation

4.1.1. Upper segments

Abscising explants exhibited a marked drop in the rate of respiration during incubation. This apparently was due to reduction in the cytochrome path activity (alternate path being not engaged at any stage and the residual respiration not changing significantly).

Although the endogenous sugar concentrations in the tissue were lower close to abscission (when compared to non-abscising controls), the reduction in respiration could not have been solely due to the limitation of respiratory substrates, since sucrose or malate could not stimulate respiration by more than 10% when added alone. In contrast, non-abscising explants (which contained a higher concentration of soluble sugars than the former), demonstrated a marked stimulation of respiration (25-35%) in response to sucrose or malate. The rate of respiration in abscising

explants could, however, be stimulated markedly by uncoupler, even in the absence of externally added substrates, which indicates that respiration was limited by the turnover of ATP rather than the availability of carbohydrates. Adenylate control of respiration can occur either via its restriction of oxidative phosphorylation in the cytochrome path, or by deregulating glycolysis. Uncoupler thus lower the cytosolic ATP:ADP ratio which leads to enhanced glycolytic activity and subsequently increased TCA cycle turnover (Day *et al.* 1980). However, in abscising segments the availability of glycolytic end products may not have been the major limitation since malate (or succinate) failed to stimulate respiration significantly more than did sucrose (malate metabolism does not involve glycolysis). Thus, adenylate control of cytochrome path appears to be the major controlling factor of respiration in these segments. The results also show that CCCP was able to increase the electron flux through the alternative path in addition to that through the cytochrome path. This suggests some control by adenylates in the respiratory path between glycolysis and ubiquinone (Bingham and Farrar 1988); the most likely point of this control is complex I (first energy transduction site) of the respiratory chain (see Fig. 1).

Control explants (at day 3) and the explants before culture, on the other hand, showed a greater stimulation by malate than sucrose, suggesting that deregulation of glycolysis in response to CCCP would have played a greater role in the increase in respiration observed upon addition of that compound than when compared to abscising explants.

The capacity of the cytochrome path also dropped very rapidly in abscising segments. This capacity is presumably determined by the carrier capacity of a

component in the cytochrome path (Blacquiere and de Visser 1984). Cytochrome c oxidase has been reported to be a rate limiting step in the electron transport chain of uncoupled sycamore protoplasts (Journet *et al.* 1986), but the apical segments of both abscising and non-abscising explants studied here did not show a reduction in cytochrome oxidase activity (in fact, it had increased to some extent by day 3).

By day 5 (after abscission) the respiration of upper segments of abscising explants could not be stimulated significantly even with the addition of the uncoupler and substrates. Electron microscopical examination at this stage showed the mitochondria in most cells to be in various stages of degradation (Figs 10b, 10c). The gradual loss of this mitochondrial integrity could account for the drop in cytochrome path activity observed. Loss of TCA cycle enzymes may have occurred under these conditions.

4.1.2. Basal segment

The increase in respiration observed in basal segments of abscising explants appeared to be due to an increase in the activity of the cytochrome pathway, close to abscission (day 3-4). The alternative pathway, despite its increased capacity close to abscission, was not engaged at any stage. However, it is not certain whether it was not operative at least for a short period of time since,

(1) for each respiratory measurement at least 2-3 explants had to be combined which might not have had perfectly synchronised abscission.

(2) the peak of ethylene production in abscising explants (Chapter 1) occurred during a very short space of time, which might also be true to the engagement of alternative oxidases.

Although cytokinins have been reported to be effective inhibitors of alternative pathway in some tissues and mitochondria (Tetley and Thimann 1974; Millar 1980, 1982; Dizengremel *et al.* 1982), it is unlikely that the zeatin in one of the abscission inducing media influenced the alternative path activity in the explants studied here, since there was no discernible difference in alternative path operation between IAA + zeatin medium and IAA only medium.

The addition of uncoupler (alone or with a substrate) resulted in increased participation of the alternative path, when compared to the controls and explants before culture (shown by the greater inhibition of SHAM), in addition to the stimulation of cytochrome path. Substrates (sucrose and malate) alone were not able to stimulate respiration significantly (5-7%) as to be expected (basal segment being in contact with the medium containing sucrose), and again the response to malate was similar to sucrose. These observations indicate that the control by adenylates is more important than the limitation of glycolytic end products in these tissues. The marked stimulation in alternative path activity by CCCP (the magnitude of its engagement being greater than in the upper segment) and the inability of exogenous malate to stimulate respiration markedly, seem to suggest some adenylate control after glycolysis but before ubiquinone (the branch point of the alternative and the cytochrome path), in addition to its control of cytochrome path (Bingham and Farrar

1988). This control could be at the site one of oxidative phosphorylation in the complex I of the respiratory chain.

The capacity of the cytochrome path did not change significantly during incubation in both abscising and non-abscising explants (except in controls at day 5 which demonstrated a decrease). The activity of cytochrome oxidase was also not significantly different from that of upper segments. Again, this enzyme demonstrated a slight increase on day 3-4 of incubation. Ultrastructural observations also revealed that most of the mitochondria were intact and had normal well developed cristae at this stage. Some cells did show increasing numbers of mitochondria (Fig. 11a), but these observations cannot be considered conclusive without more elaborate studies with serial sectioning and mitochondrial counts.

Thus, from the results discussed above, it seems that the respiratory increase in the basal segment of abscising explants was due to an increase in the cytochrome path activity which operates at 78% to 88% of its capacity (in the explants pre-culture it operates around 50-60% of its capacity). This increase could arise from the ample supply of sucrose to the basal segment.

The basal segments had marginally higher total sugar concentrations (by 2-3 days after explanting) in abscising explants compared to the controls. Whether this increase was due to hormone promoted metabolite transport (e.g. by IAA; Patrick and Steains 1987), from the upper parts of the explants (which exhibited a rapid drop in sugar concentration), or to better uptake and retention of sugars from the medium, is uncertain.

4.2. Relation of Respiration to Abscission and Senescence

Explants containing normal abscission zones have been demonstrated to exhibit climacteric-like rise in respiration during abscission (Carns 1951; Addicott 1982). This increase in respiration was prominent in the abscission zone region (Marynick 1977). In the adventitious abscission of *Impatiens sultani*, however, the precise location of the abscission layer cannot be predicted and hence the region containing the future abscission layer would contain a significant proportion of the region above the abscission layer, in the experiments described here. This could have clouded the determination of the exact respiratory response of the abscission layer cells. However, the region immediately below the abscission zone did show an increase in respiration close to abscission, and this coincided with the increase in ethylene production (Chapter 1). Ethylene has been demonstrated to be responsible for stimulating respiration in a climacteric manner in a number of tissues (Solomos and Laties 1974b, 1975).

From ethylene inhibitor studies carried out on *Ipomoea batatas* adventitious abscission (Chapter 2), it can be proposed that the basal part of the explants is more responsible (than the upper parts) for the increase in ethylene production, and that the enhancement of respiration observed in *Impatiens sultani* basal segments during abscission could be initiated by the increase in ethylene production in this region at that time.

The enhancement of respiration in response to ethylene has been reported to be due to a preferential rise in the alternative path operation, in addition to the cytochrome path (Theologis and Laties 1978; Eshashi *et al.* 1987). In adventitious

abscission studied here, the increase in respiration in the basal segment was solely due to an increase in the cytochrome path activity (although the capacity of the alternative path increased at this stage, it was not operative).

Although similar increases in respiratory activity have been reported for senescing detached fruits and leaves (Solomos and Laties 1974a; Romani 1987), the upper part of the abscising explants did not demonstrate such a phenomenon. Instead, the rate of respiration dropped rapidly; this was partly due to respiratory control by adenylates during the initial stages, and then due to the loss of mitochondrial integrity during the latter stages (as shown by ultrastructural studies). A respiratory climacteric appears in the senescence of many intact or detached plant organs, but it is not true for the senescence of all plants (Thimann 1980). Although the seemingly non-senescing basal segments (in abscising explants) demonstrated such a phenomenon, the senescing upper segments of *Impatiens sultani* might belong to the latter category. Thimann (1980) also reported that the respiratory climacteric is more prominent in detached organs which is supposed to be due to transport of metabolites (e.g. respirable material) out of the organ. This might have also played some role in the adventitious abscission studied here, where transport from the senescing upper parts to the lower non (or slow) senescing segment could occur.

Table 1: Changes in the rate of oxygen uptake of different segments of explants cultured in two abscission inducing media (0.0001% IAA +1.0% sucrose and 0.0001% IAA + 0.0001% zeatin + 1.0% sucrose). The segments (each 5.0mm long) are numbered starting from the upper end of the explant (see diagram below this table)

Medium		Explant segment	nmol $O_2.g.^{-1}f.Wt.min^{-1}$					
IAA Conc. (%)	Zeatin Conc. (%)		Pre-culture	Day 2	Day 3	Day 4	Day 5	Day 6
0.0001	-	1	83.1	60.5	42.0	30.8	21.9	14.8
		2		62.5	51.0	40.2	30.0	19.8
		3		69.5	70.8	65.2	58.2	39.5
		4	80.5	78.2	82.1	88.9	74.1	68.0
0.0001	0.0001	1	79.8	55.2	40.0	22.8	18.2	14.5
		2		60.1	49.0	31.2	20.2	17.8
		3		70.4	79.2	59.0	48.0	38.5
		4	82.2	80.2	95.1	80.0	74.5	71.8

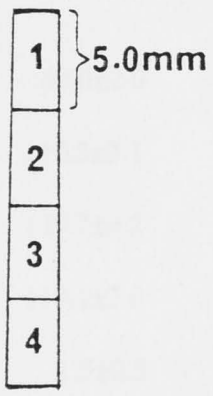


Table 2: Effect of various compounds on rate of oxygen uptake of **upper segments** (segment 1 and 2 combined) of the explants, pre- culture (initially) and at two stages of incubation (day 3 and 5). Media used were 0.0001% IAA + 0.0001% zeatin + 1.0% sucrose and 1.0% sucrose (control). The sequential additions as indicated were 1.0 µM CCCP, 5.0 mM SHAM, 0.3 mM KCN, 100 mM Malate and 100 mM sucrose. The values are means of 2-3 measurments ± S.E., with 2-3 explants per measurement

	Sequential additions	O_2 uptake (nmol $O_2.g.^{-1}f.wt.min^{-1}$)				
		Pre-culture	Day3		Day5	
			IAA+Zeatin	Control	IAA+Zeatin	Control
Exp.1	None	79.2±3.1	44.4±2.5	52.0±3.3	20.0±1.0	40.0±1.3
	Malate	94.5±3.8	49.1±2.0	70.0±3.8	29.1±1.0	50.1±1.0
	CCCP	132.6±4.0	93.4±3.8	114.1±4.9	35.2±1.2	79.9±1.8
	SHAM	118.4±2.2	75.7±2.3	81.8±3.2	32.0±0.5	60.1±1.1
	KCN	1.1±0.2	0.0	0.0	0.0	0.0
Exp.2	None	78.3±1.8	48.1±2.2	55.0±2.0		
	Sucrose	88.2±2.2	52.2±2.1	68.8±2.4		
	CCCP	121.6±3.8	91.4±3.1	110.3±3.5		
	SHAM	113.3±2.9	76.0±3.2	88.1±2.0		
	KCN	1.2±0.4	0.0	0.0		
Exp.3	None	80.0±2.0	42.5±2.0	50.0±1.9		
	CCCP	110.5±3.1	85.5±3.2	82.8±1.8		
	Malate	120.7±4.2	91.3±3.1	100.1±2.0		
	SHAM	110.1±3.0	70.1±2.3	82.0±1.0		
	KCN	1.5±0.5	0.0	0.0		

Table 3: Effect of various compounds on rate of oxygen uptake of **basal segment** (segment 4) of the explants, at two stages of incubation (day 3 and 5). Media used were 0.0001% IAA + 0.0001% zeatin + 1.0% sucrose and 1.0%sucrose (control). The sequential additions as indicated were 1.0 µM CCCP, 5 mM SHAM, 0.3 M KCN, 100 mM Malate, 100 mM sucrose. The values are means of 2-3 measurments ±S.E., with 2-3 explants per measurement

	Sequential additions	O_2 uptake (nM $O_2.g.^{-1}f.Wt.min^{-1}$)			
		Day3		Day5	
		IAA+Zeatin	Control	IAA+Zeatin	Control
Exp.1	None	85.1±4.3	70.0±3.0	68.1±3.2	62.3±2.5
	Malate	90.5±4.0	74.2±2.9	70.4±2.8	64.8±2.1
	CCCP	150.8±6.1	140.1±3.3	125.8±3.9	106.4±3.5
	SHAM	95.9±3.9	90.1±2.1	95.1±2.2	72.0±2.0
	KCN	3.0±1.0	4.9±0.8	0.0	0.0
Exp.2	None	87.8±5.1	74.1±3.0		
	Sucrose	92.7±5.0	79.3±3.1		
	CCCP	177.5±8.5	152.6±3.9		
	SHAM	104.2±4.0	96.6±2.3		
	KCN	5.2±1.1	4.0±1.3		
Exp.3	None	81.9±4.9	70.1±2.8		
	CCCP	140.2±5.8	125.1±3.3		
	Malate	148.5±5.0	132.8±3.1		
	SHAM	100.2±4.2	92.1±3.0		
	KCN	0.0	0.0		

Table 4: The cytochrome oxidase activity (expressed as µmol Cytochrome c oxidized $min.^{-1}g.^{-1}$f.Wt.) of the upper and the basal segments at different stages of incubation. The results are combined measurements of 3-4 explants. The figures within parentheses are the rates of cytochrome c oxidation after addition of 5 mM KCN to inhibit cytochrome oxidase activity. Media used were 0.0001% IAA + 0.0001% zeatin +1.0% sucrose and 1.0% sucrose (control).
Cytochrome oxidase activity of explants pre-culture (initial) were 49.2 and 55.0 µmol Cyt.c $oxidized.min^{-1}.g.^{-1}$f.Wt. in segments 1+2 and 4 respectively

Medium	Explant segment	µmol Cyt.c $oxidized.min^{-1}.g.^{-1}$f.Wt					
		Day0.5	Day1	Day2	Day3	Day4	Day5
IAA+Zeatin	1+2	50.7	55.1	61.0	75.0	60.1	54.0
						(20.1)	(19.0)
	4	54.1	62.0	60.3	78.9	70.5	62.8
						(18.2)	(15.1)
Control	1+2	60.0	62.4	59.0	69.0	70.1	
					(15.1)	(24.1)	
	4	55.7	58.0	55.3	71.1	69.5	
					(20.0)	(17.5)	

Table 5: Changes in total sugar content (mg.g.$^{-1}$f.Wt.) in different segments of the explant during incubation. The results shown are combined measurements of 2-4 explants. Media used were 0.0001% IAA +1.0% sucrose, 0.0001% IAA + 0.0001% zeatin +1.0% sucrose and 1.0%sucrose (control). Total sugar content of explants pre-culture (initial) were 6.68, 6.58, 6.19 mg.g.$^{-1}$f.Wt. in segments 1+2, 3 and 4 respectively

Medium	Explant segment	Total sugar content(mg.g.$^{-1}$f.Wt.)				
		6h	15h	32h	62h	84h
IAA	1+2	6.29	5.05	3.54	1.24	0.69
	3	6.22	6.48	5.65	4.20	2.66
	4	6.38	7.17	7.59	8.32	8.86
IAA+Zeatin	1+2	5.97	5.91	3.65	1.84	0.38
	3	6.20	6.46	5.17	4.63	1.7
	4	6.93	8.13	8.68	8.31	7.23
Control	1+2	5.30	5.20	4.09	2.89	1.98
	3	5.95	5.33	4.37	4.18	4.12
	4	6.35	6.69	6.94	7.17	7.78

Fig. 1. Higher plant respiratory chain. The known components are arranged into their presumed complexes (Moore and Rich 1985), and sites of inhibitor action (rotenone, antimycin, KCN,and SHAM) are denoted by bold lines.

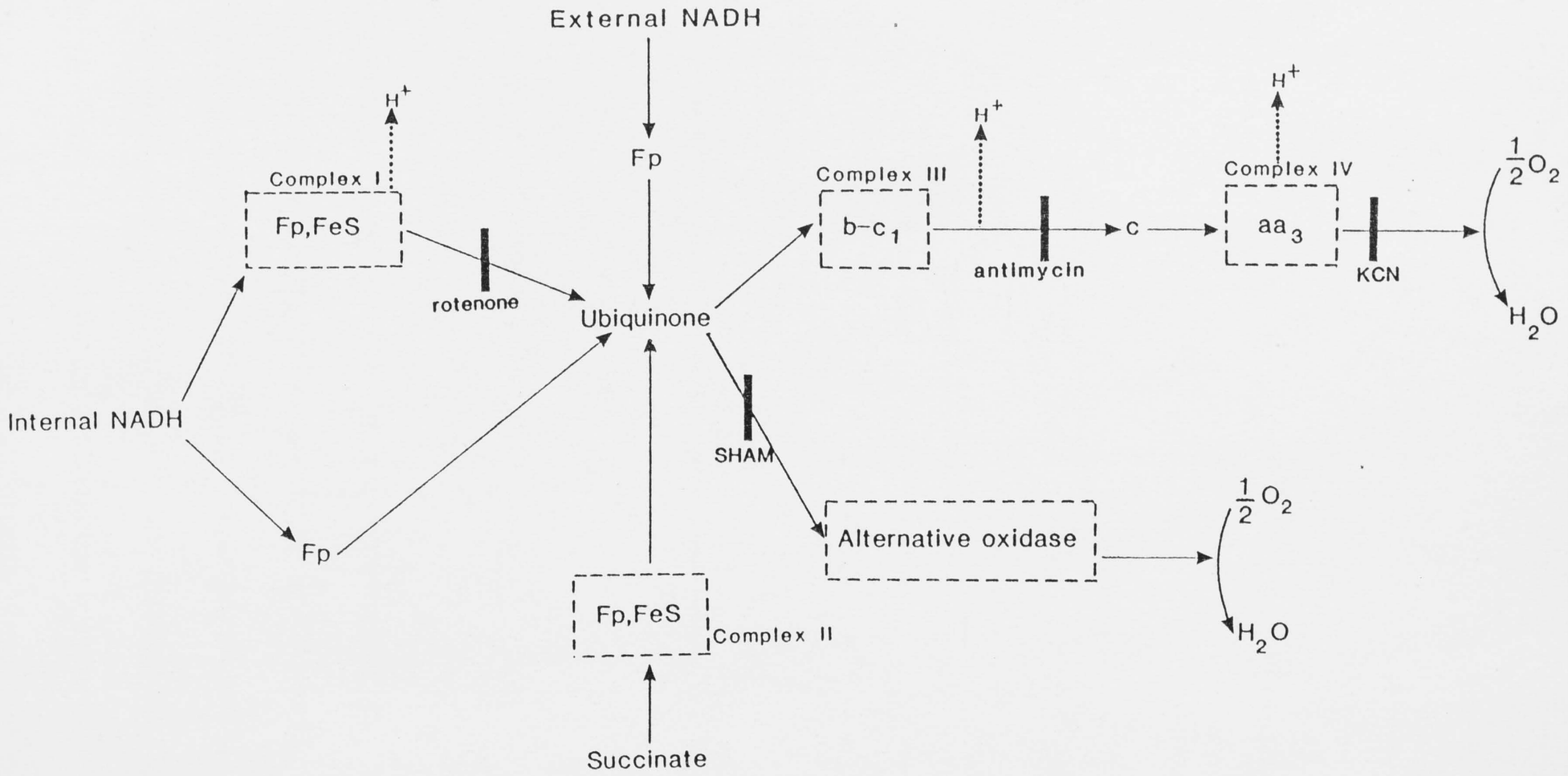

External NADH
Fp
H+
Complex I
Fp,FeS
rotenone
Ubiquinone
Complex III
b-c1
H+
antimycin
c
Complex IV
aa3
H+
KCN
1/2 O2
H2O
Internal NADH
Fp
SHAM
Alternative oxidase
1/2 O2
H2O
Fp,FeS
Complex II
Succinate

Fig. 1.

Fig. 2. A simplified scheme of respiration and its control points adopted from Lambers (1985). The controlling factors include concentration of substrates (1) adenylates (which control via constrains on oxidative phosphorylation (2) by inhibiting glycolysis(3)), the alternative path(4) which becomes engaged when the capacity of the cytochrome path is exceeded by high electron flux. The rotenone insensitive by-pass (5) (Fig. 1) presumably operates when the rate of endogenous NADH production is high.

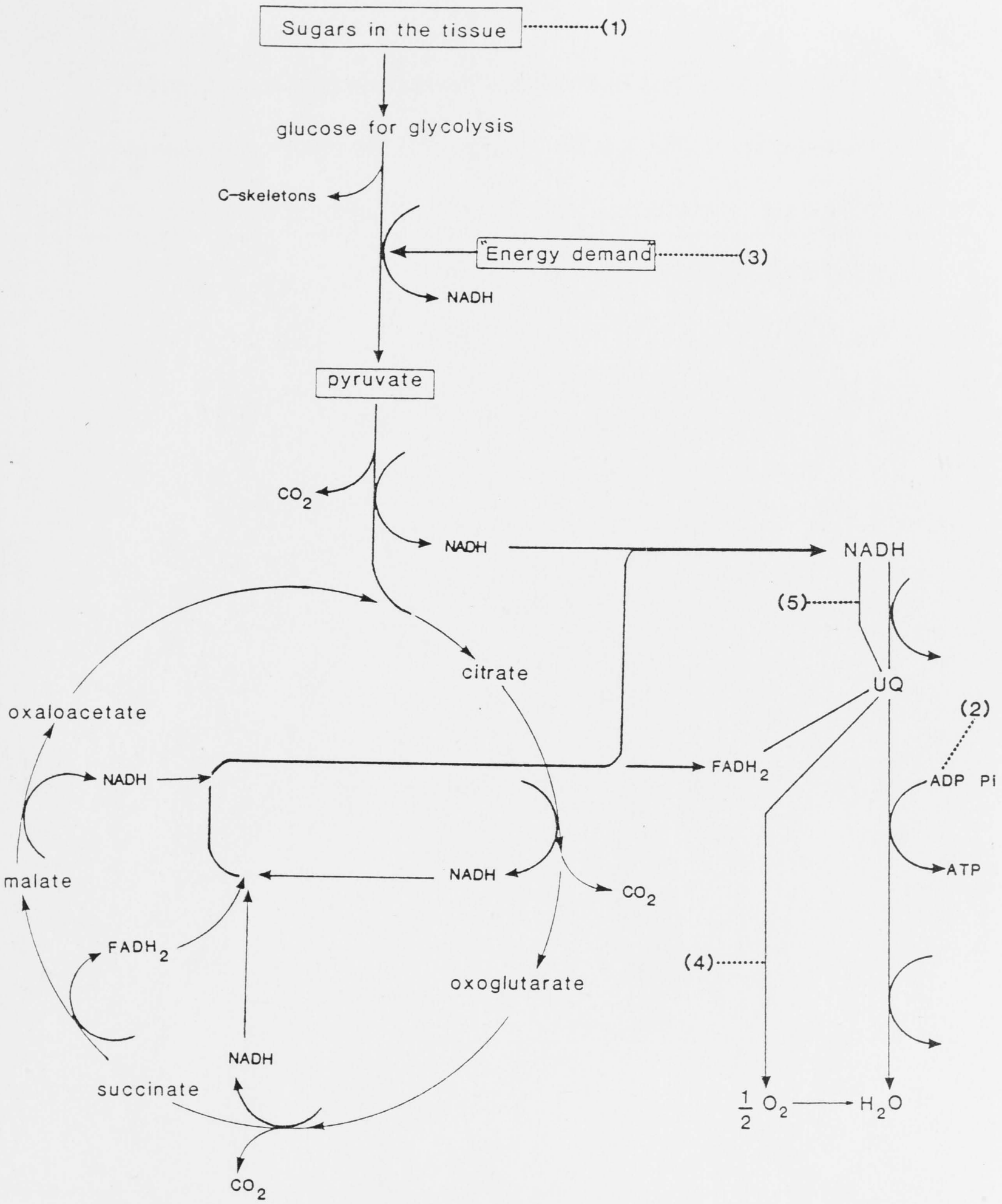

Sugars in the tissue
(1)
glucose for glycolysis
C–skeletons
"Energy demand"
(3)
NADH
pyruvate
CO_2
NADH
NADH
(5)
citrate
UQ
(2)
oxaloacetate
NADH
$FADH_2$
ADP Pi
malate
NADH
ATP
CO_2
$FADH_2$
(4)
oxoglutarate
NADH
succinate
$\frac{1}{2}$ O_2 → H_2O
CO_2

Fig. 2.

Fig. 3. The titration of fresh stem slices of *Impatiens sultani* with different concentrations of SHAM in the presence of 0.3mM KCN. The results are expressed as percentage inhibition of KCN inhibited O_2 uptake versus SHAM concentration used.

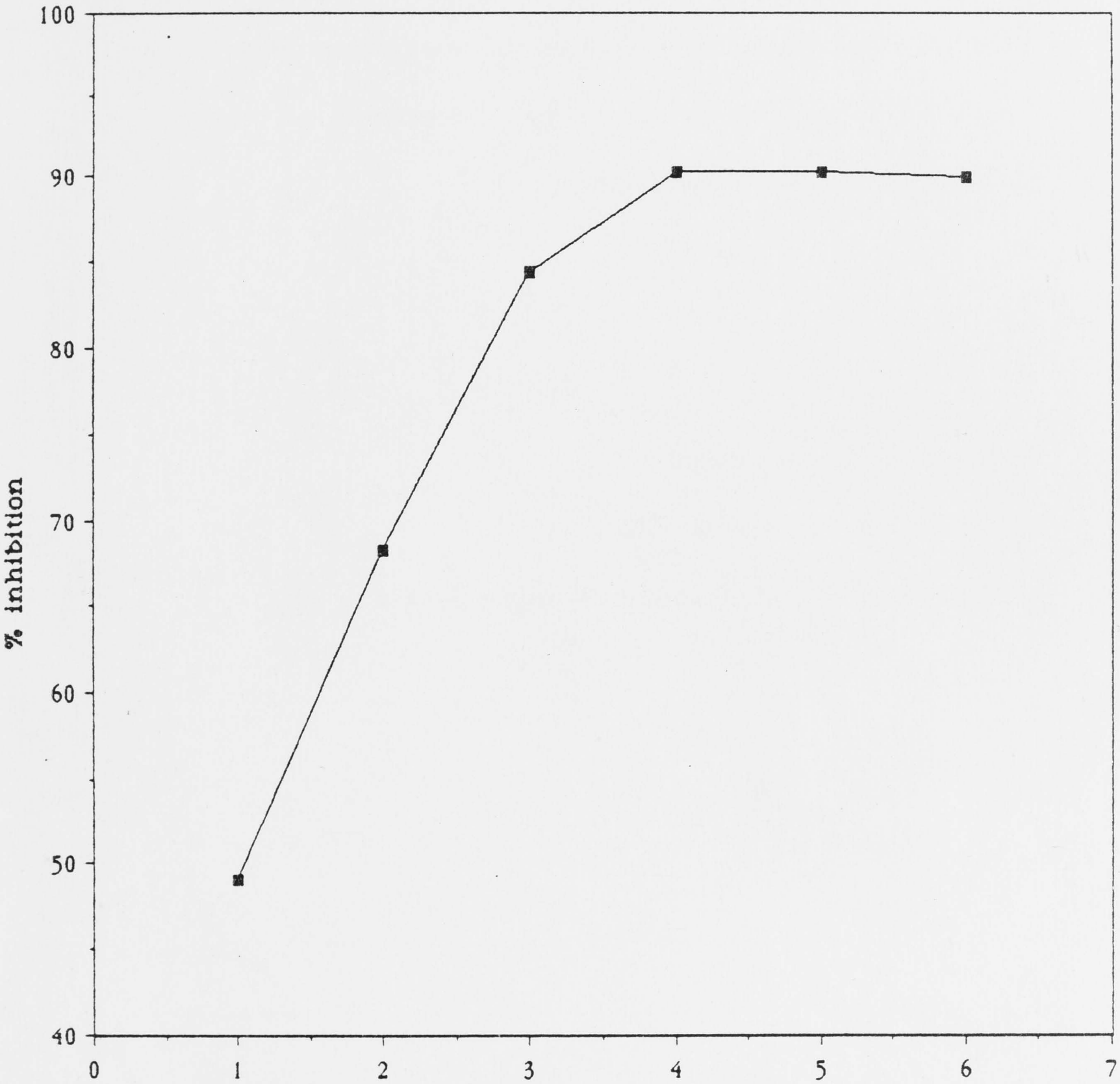

100
90
80
70
60
50
40
% inhibition
0
1
2
3
4
5
6
7
SHAM concentration (mM)

Fig. 3

Fig. 4. Changes in the rate of respiration of upper segments (segment 1 + 2) of explants during incubation in 2 abscission inducing media (0.0001% IAA + 1.0% sucrose and 0.0001% IAA + 0.0001% zeatin + 1.0% sucrose) and control media (1.0% sucrose). Arrows indicate time of abscission. (Figs 4-6 are from the same set of experiments, and values shown are means of 2-3 experiments)

□ 1% sucrose medium (control)

△ 0.0001% IAA + 1% sucrose medium

▲ 0.0001% IAA + 0.0001% zeatin + 1% sucrose medium

Oxygen uptake (nmol O_2/g.f.Wt./min.)

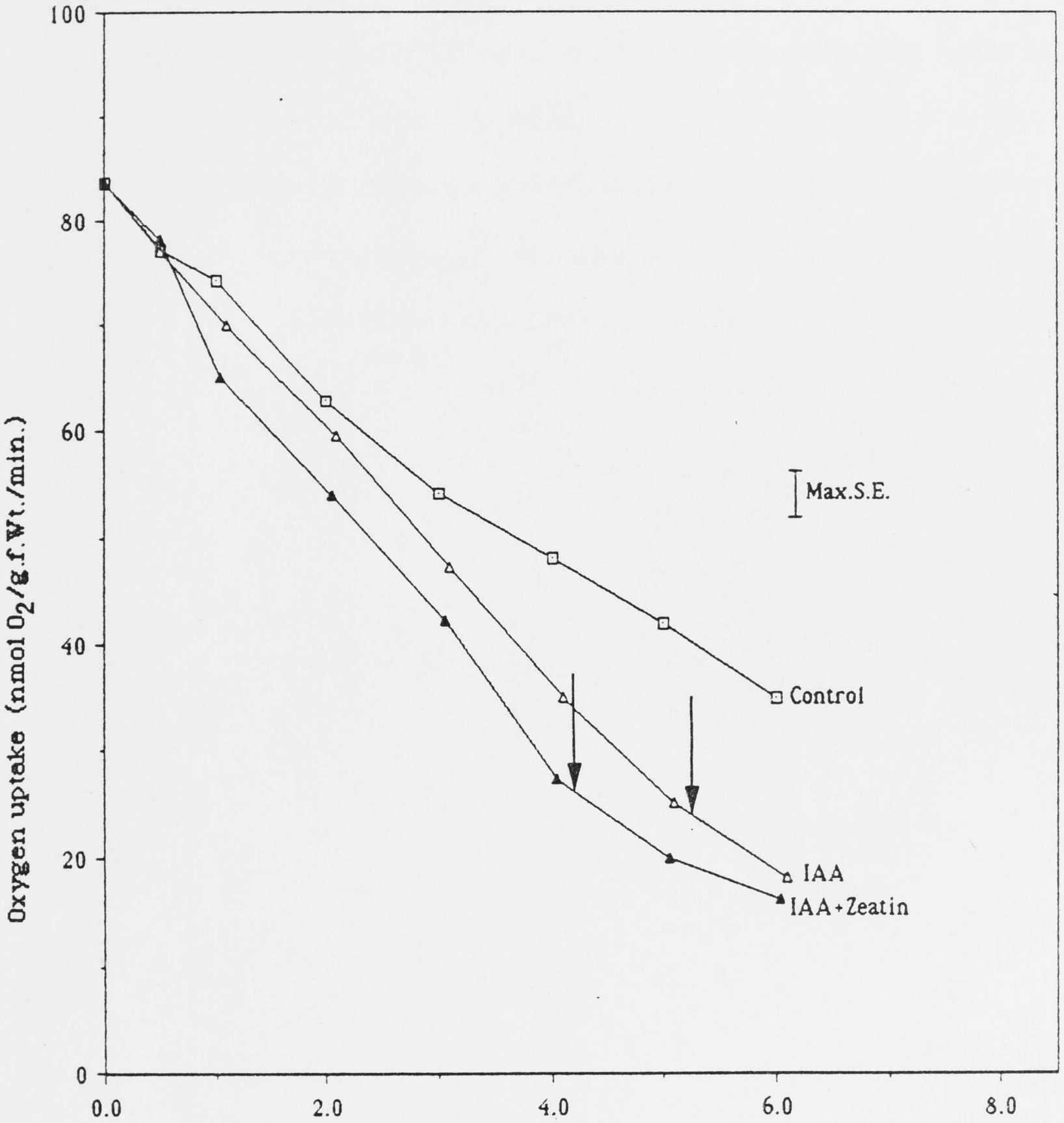

100
80
60
40
20
0
Oxygen uptake (nmol O_2/g.f.Wt./min.)
Max.S.E.
Control
IAA
IAA+Zeatin
0.0
2.0
4.0
6.0
8.0
Days after explanting

Fig. 4

Fig. 5. Changes in the activity of the cytochrome path (v_{cyt}) of the upper segments (segments 1 + 2) during incubation of the explants in the 2 abscission inducing and control media (see legend of Fig. 4). The activity of the cytochrome path was estimated by substracting the activity of the alternative path (v_{alt}, which was zero during the entire period of incubation) and residual respiration (v_{res}, which is the respiration resistant to 0.3mM KCN and 5mM SHAM), from O_2 uptake in the absence of inhibitors (see Fig. 4).

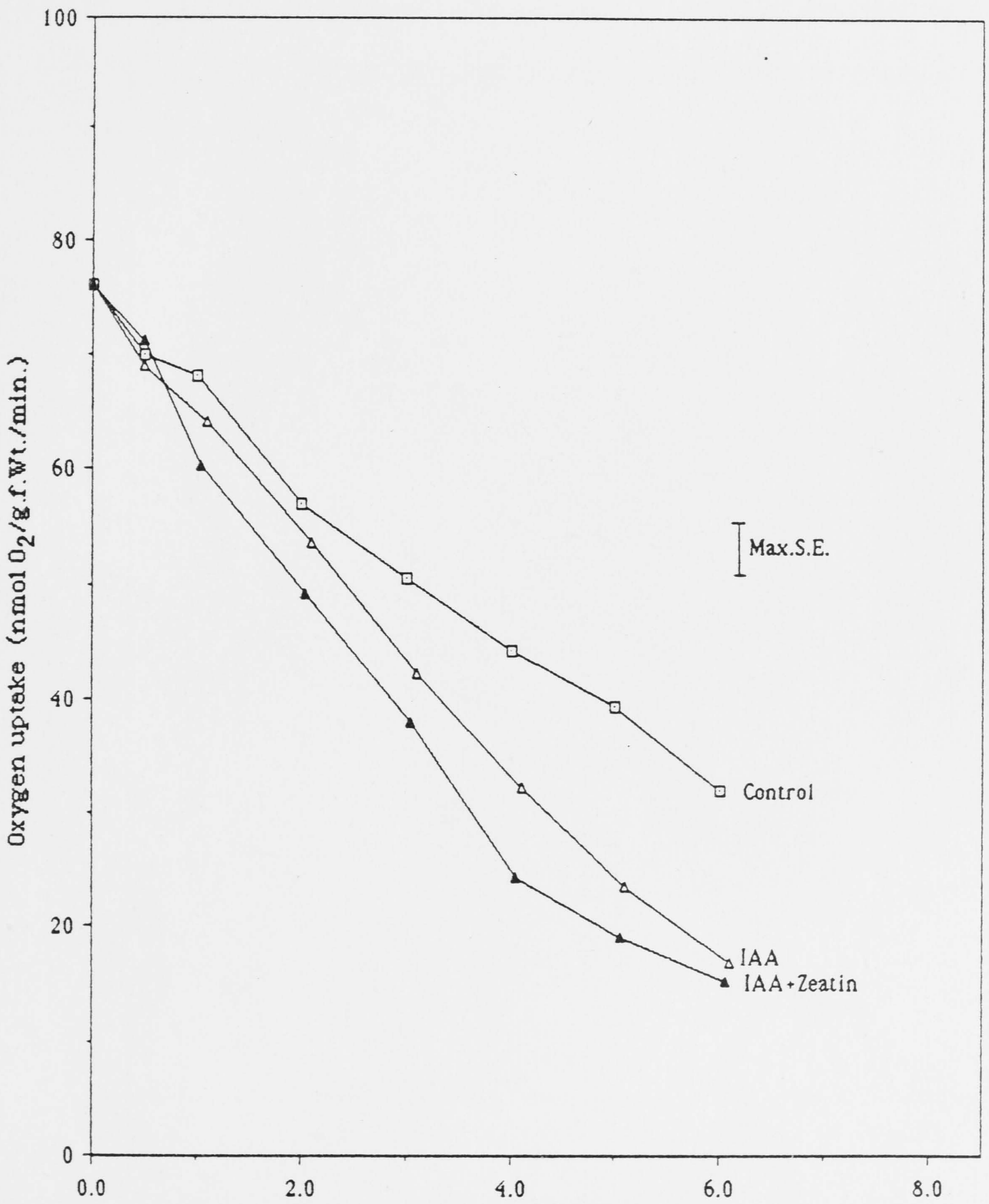

100
80
60
40
20
0
Oxygen uptake (nmol O_2/g.f.Wt./min.)
Max.S.E.
Control
IAA
IAA+Zeatin
0.0
2.0
4.0
6.0
8.0
Days after explanting

Fig. 5

Fig. 6. Changes in the capacity of the alternative oxidase (V_{alt}) during incubation of explants in 2 abscission inducing media and control medium (see legend of Fig. 4), estimated as the rate of O_2 uptake resistant to 0.3mM KCN minus the residual respiration.

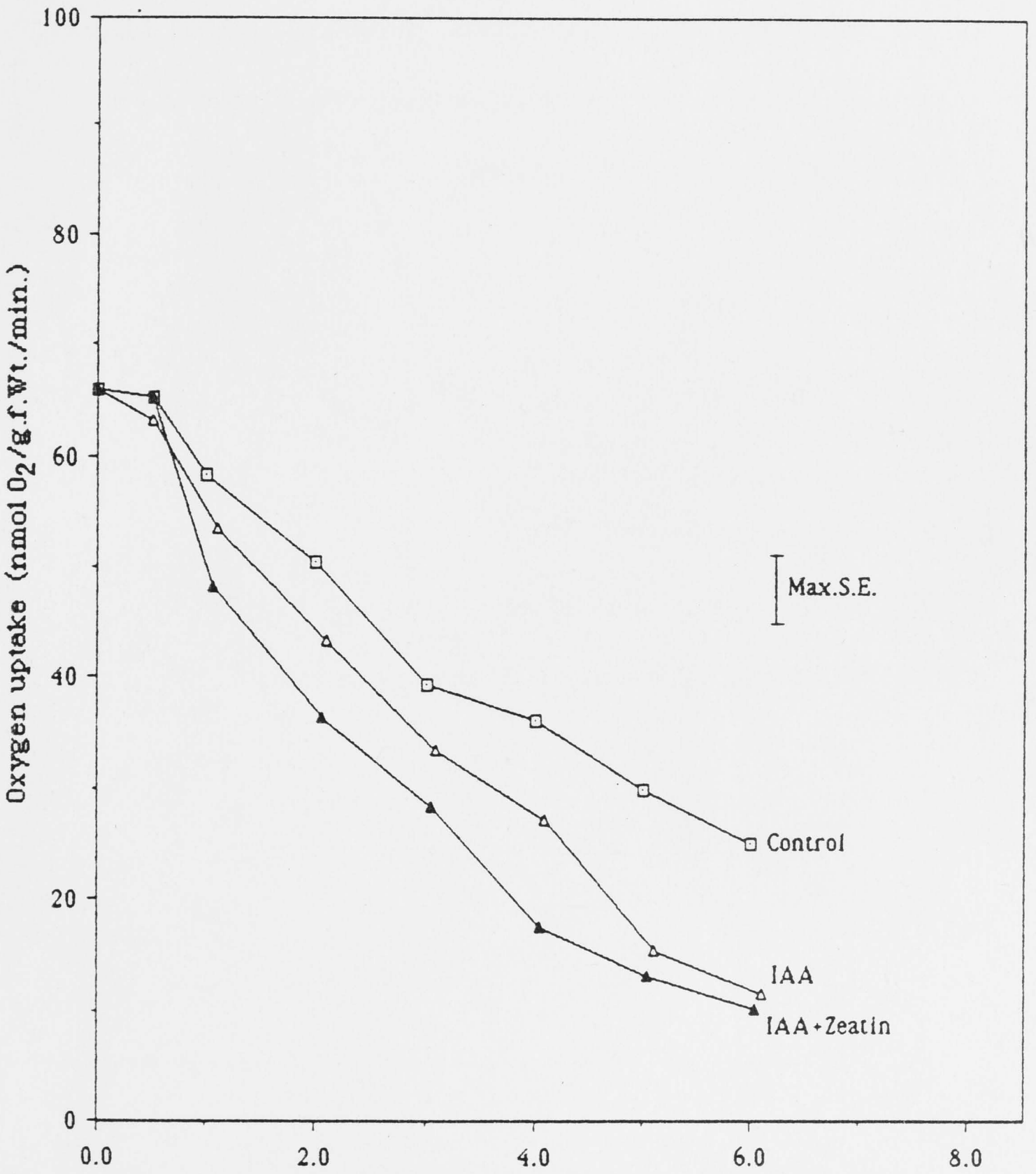

100
80
60
40
20
0
Oxygen uptake (nmol O2/g.f.Wt./min.)
0.0
2.0
4.0
6.0
8.0
Days after explanting
Max.S.E.
Control
IAA
IAA+Zeatin

Fig.6

Fig. 7. Changes in the rate of respiration of the basal (lower) segment (segment 4) of the explants during incubation in 2 abscission-inducing media (0.0001% IAA + 0.0001% zeatin + 1% sucrose and 0.0001% IAA + 1% sucrose) and control medium (1% sucrose). Arrows indicate time of abscission. Figs 7-9 are from the same set of experiments as Figs 4-6 and are means of 2-3 experiments.

□ 1% sucrose medium (control)

△ 0.0001% IAA + 1% sucrose medium

▲ 0.0001% IAA + 0.0001% zeatin + 1% sucrose medium

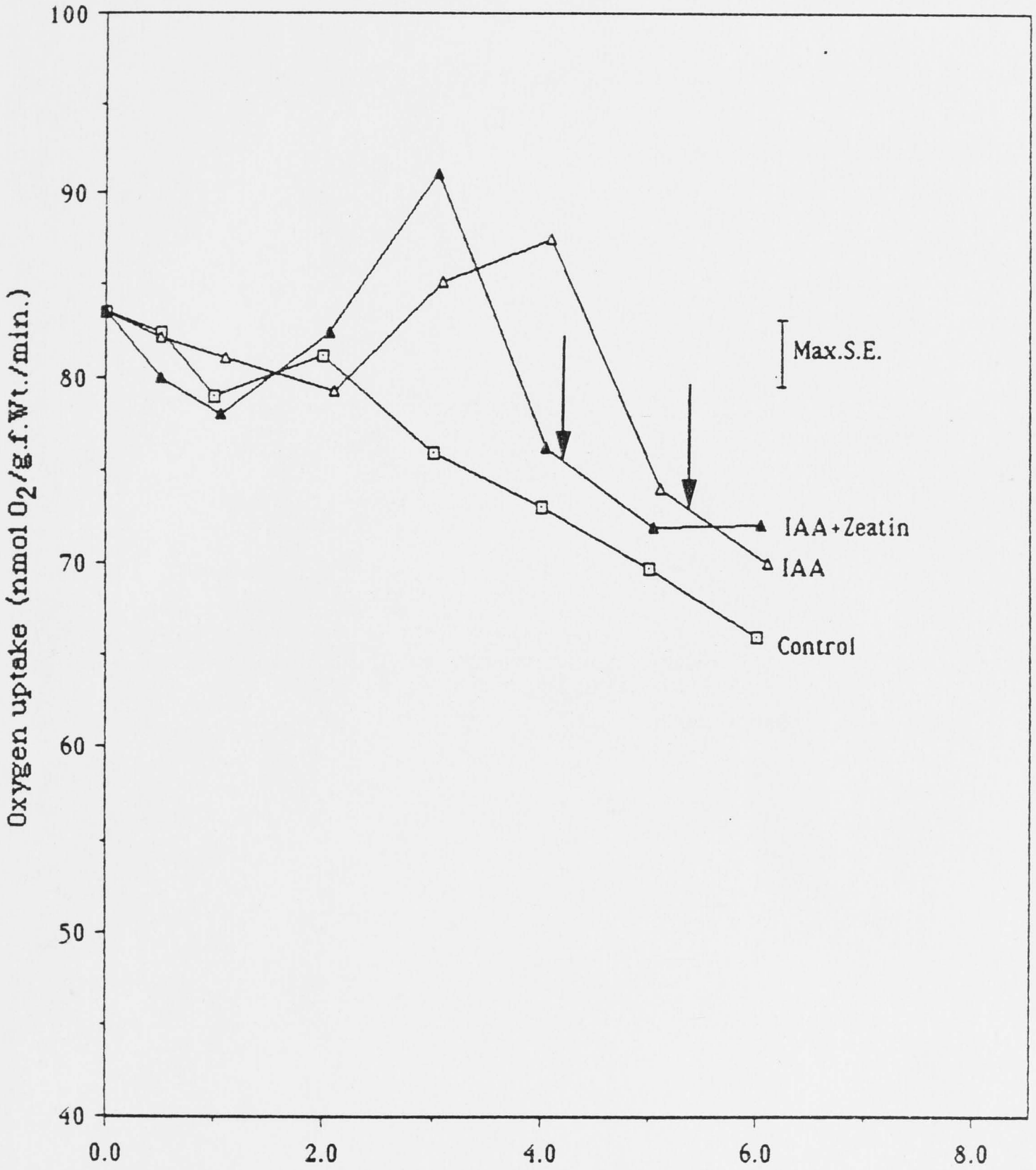

100
90
80
70
60
50
40
0.0
2.0
4.0
6.0
8.0
Oxygen uptake (nmol O_2/g.f.Wt./min.)
Max.S.E.
IAA+Zeatin
IAA
Control
Days after explanting

Fig. 7

Fig. 8. Changes in the activity of the cytochrome path (v_{cyt}) of basal segment of explants during incubation in 2 abscission inducing media and control medium (see legend of Fig. 7). The activity of the cytochrome path was estimated as described in Fig. 5. The activity of the alternative path was again zero.

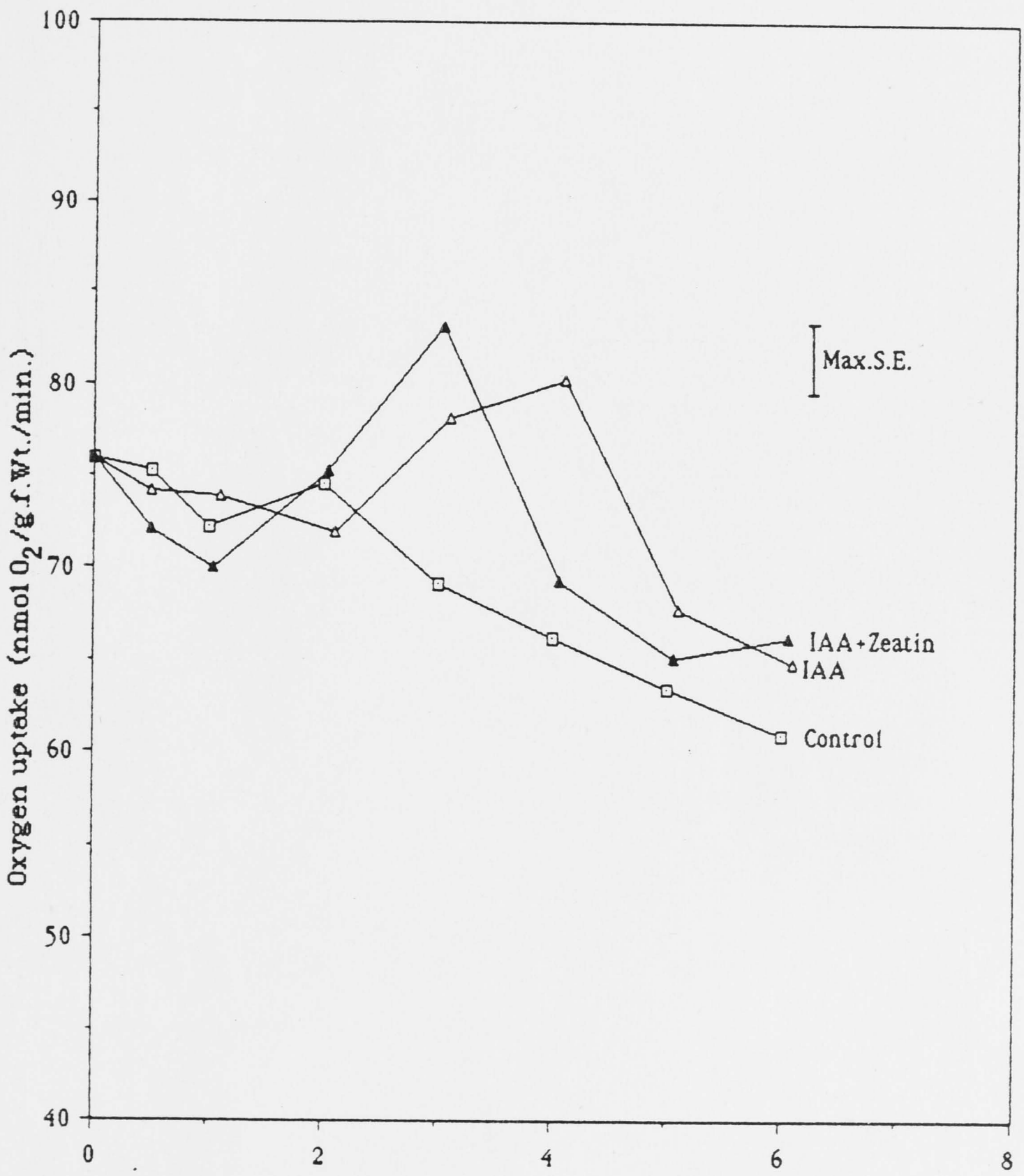

100
90
80
70
60
50
40
0
2
4
6
8
Oxygen uptake (nmol O_2/g.f.Wt./min.)
Max.S.E.
IAA+Zeatin
IAA
Control
Days after explanting

Fig. 8

Fig. 9. Changes in the capacity of the alternative oxidase (V_{alt}) in basal segments of explants during incubation in 2 abscission inducing media and control medium (see legend to Fig. 7), estimated as described under Fig. 6.

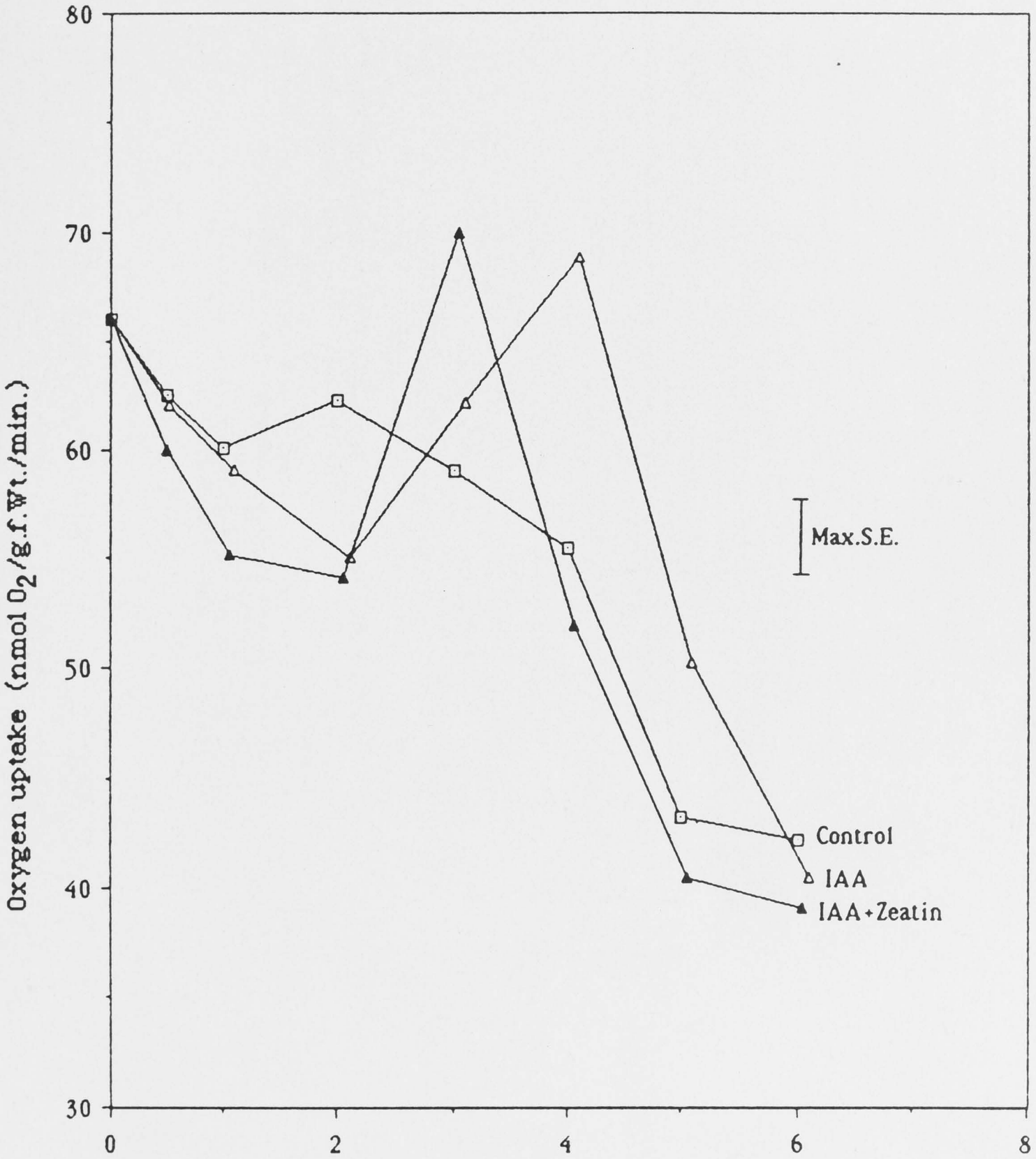

80
70
60
50
40
30
0
2
4
6
8
Oxygen upteke (nmol O_2/g.f.Wt./min.)
Max.S.E.
Control
IAA
IAA+Zeatin
Days after explanting

Fig. 9

Figs 10a-10c. Electron micrographs of a cortical cell in the upper segment 5 days after explanting (immediately after abscission).

Fig. 10a. Note the reduced cytoplasmic density and the vesiculated nature of the cytoplasm.

Scale bar = 10μm.

Figs 10b-10c. Mitochondria at different stages of disintegration. Scale bar = 1μm.

Figs. 11a-11b. Electron micrographs of a cortical cell in the basal segment, 4 days after explanting.

Fig. 11a. Note the increased number of mitochondria and dense cytoplasm.

Scale bar = 2μm.

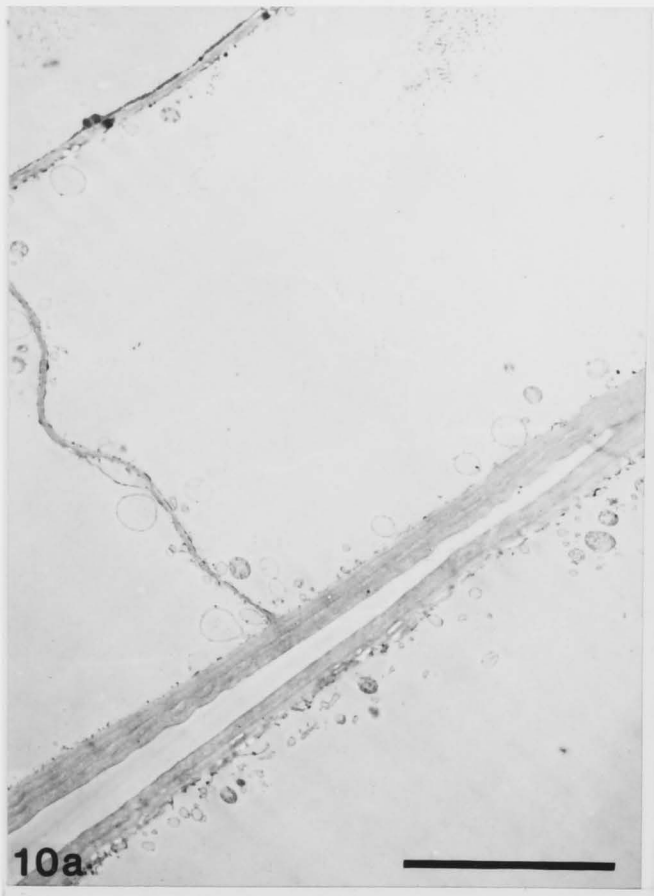
Fig. 11b. Enlarged area of Fig. 11a showing intact mitochondria with well developed cristae.

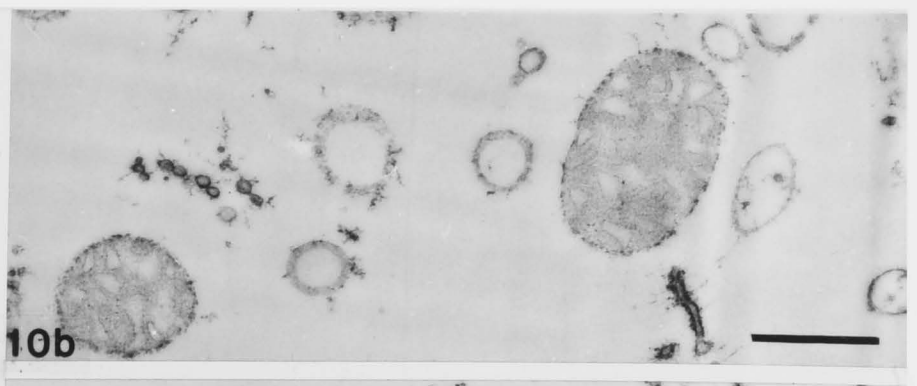
Scale bar = 1μm.

Fig. 12. Electron micrograph of the cytoplasm of a pith cell of the basal segment 5 days after explanting, showing intact mitochondria and free ribosomes in the cytoplasm. Scale bar 1μm.

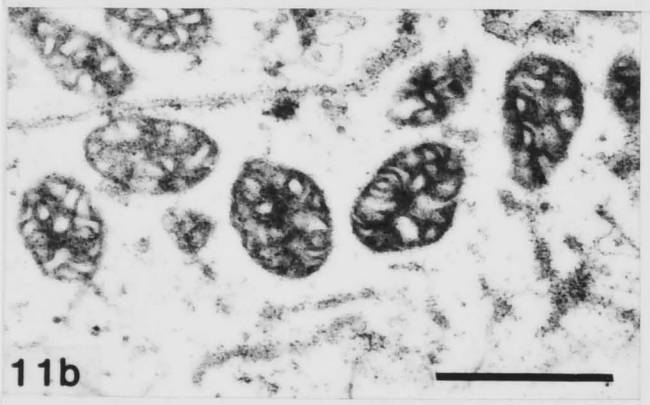
Fig. 13. A cortical cell of the basal segment 7 days after explanting. Note the increased amount of vesicles (Ve) in the dense cytoplasm.

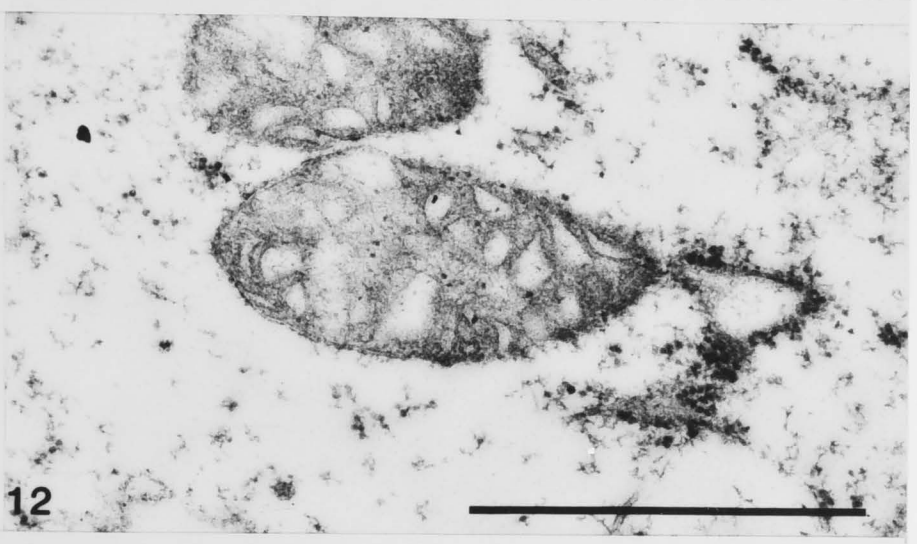
Scale bar = 1μm.

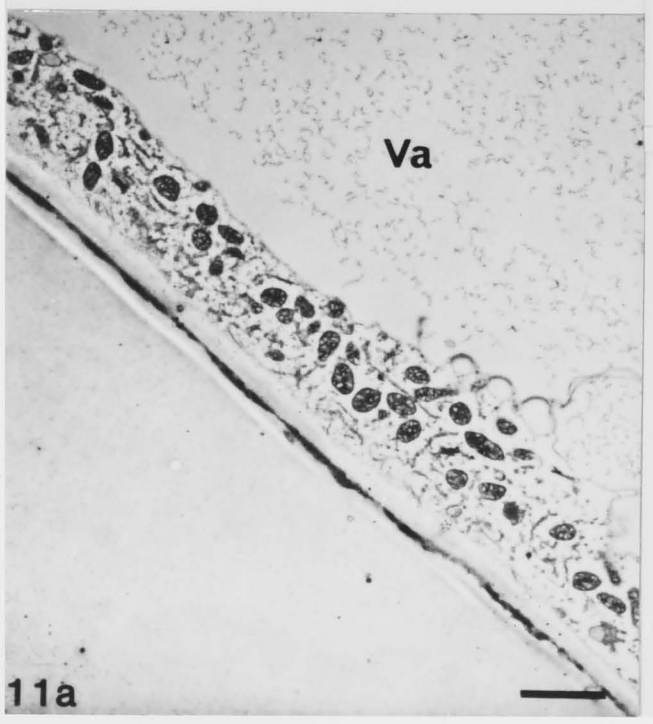

10a

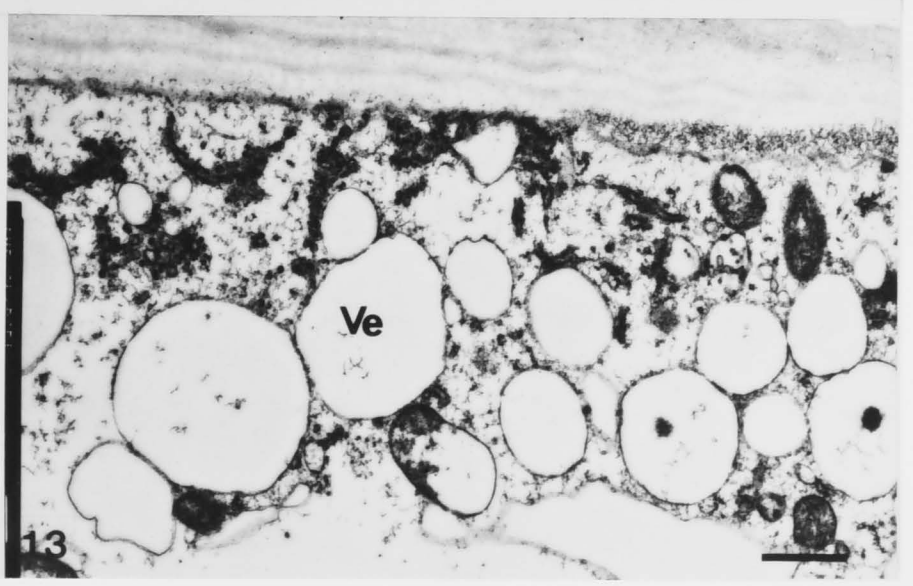

10b


10c


11b


12


Va
11a


Ve
13

GENERAL DISCUSSION

Results described in Chapter 1 revealed that zeatin acts synergistically with IAA in inducing adventitious abscission in both *Impatiens sultani* and *Ipomoea batatas* internodal explants when applied basally. Such abscission was accompanied by a peak rate of ethylene production just before abscission, which was more pronounced in explants cultured in media containing both IAA and zeatin than those in the IAA only medium. Ethylene being a known promoter of abscission, the high rate of ethylene production suggests that the abscission-stimulative effect of zeatin, when added in the presence of IAA is mediated by its ability to augment ethylene-synthetic capacity of the explants. This is probably effected via cytokinin's (zeatin's) ability to maintain high free IAA levels (see discussion of Chapter 1). This in turn could induce ACC synthase synthesis, thus stimulating ethylene production (Yu and Yang 1979; Yoshii and Imaseki 1982). The important role played by ethylene in adventitious abscission was further corroborated by the finding that ethylene alone without any other externally added hormones was capable of inducing abscission in *Impatiens sultani*, *Ipomoea batatas* and to a certain extent in *Begonia corallina*. Further evidence for the involvement of ethylene in adventitious abscission is provided in the present investigation from studies using inhibitors of ethylene synthesis (AVG), and ethylene action antagonist (Ag^{+}) on abscission of *Ipomoea batatas* internodal explants (Chapter 2). These results demonstrated that both lower and upper separation layers in *Ipomoea batatas* can be inhibited by the use of ethylene inhibitors or antagonists. The inhibitory action of the apically applied AVG on the upper separation layer could be counteracted by culturing the explants in a

medium containing higher concentration of IAA (i.e. in a medium containing 0.001% IAA as opposed to the normally used concentration of 0.0001%: Chapter 2). Explants in high IAA medium was found to produce higher levels of ethylene. Investigations employing inhibitors of ethylene also led to deduce that in *Ipomoea batatas* explants the basal part was more responsible for the production of ethylene. This might be also true for *Impatiens sultani*.

The hypothesis that the setting up of an auxin gradient is important in the positional definition of adventitious abscission (in *Impatiens sultani*: Warren Wilson 1986) was further supported by the observation that even in *Ipomoea batatas* the position of the separation was observed to form further away from the base when increasing concentrations of IAA was applied to the base. The steps following this presumably are similar to that of normal abscission of plant organs. That is, once the committed or target cells of the separation zone are sensitized, they respond to ethylene (exogenous or endogenous) by producing and secreting cellulases and other hydrolytic enzymes (Sexton *et al.* 1985).

Ethylene, in addition to acting directly on separation layer cells during adventitious abscission may also indirectly influence the abscission process by increasing auxin deactivation (Gaspar *et al.* 1978) or reducing auxin transport. The, basally applied IAA, in addition to aiding in creating an auxin gradient along the explants would also influence abscission by stimulating ethylene production (ethylene production by explants in IAA media was higher than those of the controls).

The ultrastructural investigations carried out here (Chapter 3) revealed that the separation layer cells of adventitious abscission (in *Impatiens sultani*, *Ipomoea batatas* and *Begonia corallina*) are very similar to normal abscission zone cells (e.g. abscission zone cells of leaf, fruit, or flower) in showing increased endomembrane activity prior to cell separation. *Ipomoea batatas* which exhibits cell division (unlike the other two species studied) at the separation layer prior to abscission is also comparable to normal abscission in some plant species which is accompanied by cell division (Gawardi and Avery 1950; Sexton and Roberts 1982). The anatomical and ultrastructural details of the separation layer did not differ significantly depending on the type of inductive treatment used (IAA, IAA + zeatin or ethylene), however, in ethylene induced abscission, an enlargement of the separation layer cells was apparent, especially in *Ipomoea batatas*.

Studies on the respiratory properties of adventitious abscission in *Impatiens sultani* (Chapter 4) revealed a progressive decrease in the rate of respiration in the upper segment of abscising explants. On the other hand, the basal segment was found to exhibit a respiratory increase just before abscission. Interestingly, this high rate of respiration was concurrent with a peak in ethylene production by the explants. Such climacteric like increase in respiration and ethylene production is characteristic of many abscising, ripening or senescing plant parts or organs (Solomos and Laties 1974b, Addicott 1982). The increase in respiration observed in the basal segment was found to be due to an increase in the cytochrome path activity. Although activation of the alternate path under similar circumstances in other types of tissues have been reported (Solomos and Laties 1974a), in the basal (or the upper) segments

of *Impatiens sultani* such an alternative path engagement was not observed, despite its increased capacity at that stage (Chapter 4, Fig. 9). However, this non-engagement is inconclusive due to certain practical limitations (see detailed discussion in Chapter 4).

At this stage (by 3-4 days after explanting) the upper segment showed signs of senescence both externally and ultrastructurally, unlike the basal segment which did not show such signs at the same stage. The progressive drop in the rate of respiration of the upper segment is in contrast to many senescing organs which demonstrate an increase in respiration during senescence (Tetley and Thimann 1974). The drop in respiration was found to be due to a progressive reduction of cytochrome path activity, controlled initially by adenylates, and during later stages due to the loss of mitochondrial integrity (as observed ultrastructurally).

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